Vol. XLII No. 9

September, 1953

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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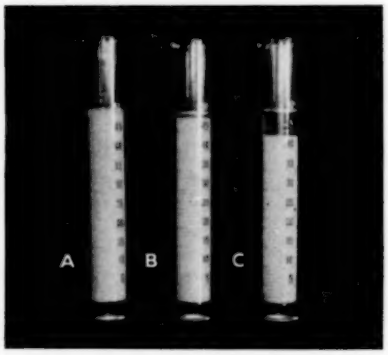

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Annual Subscription—Journal of the American Pharmaceutical Association, complete (both editions): United States and Pan America $7.00; Canada $7.70; other foreign, $8.00; members of the American Pharmaceutical Association with dues, $4.00. Each edition, Scientific Edition or Practical Pharmacy Edition: United States and Pan America, $4.00; Canada, $4.35; other foreign, $4.50. Single numbers, either edition: United States and Pan America, $0.35; Canada, $0.40; other foreign, $0.50.

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Entered as second-class matter January 23, 1917, at the Post Office at Easton, Pa., under the act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the fifth; Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLII | *SEPTEMBER, 1953* | NUMBER 9 | CONSECUTIVE NO. 18

Titration of Pharmaceuticals in Nonaqueous Solvents*

By CHARLES W. PIFER, ERNEST G. WOLLISH, and MORTON SCHMALL

THE DEVELOPMENT of new drugs in recent years has encouraged the introduction of new methods of analysis and control. Since most of these compounds are the product of organic synthesis, they have known structures and a majority of them possess characteristic groups permitting quantitative analysis. A concise treatment of this subject can be found in Siggia's book, *Quantitative Organic Analysis via Functional Groups* (75).

One difficulty frequently encountered in the analysis of organic compounds is their poor solubility and relatively weak reactivity in water. The same compounds, however, when dissolved in an organic solvent, may often show properties which permit their simple and precise determination.

Analysis in nonaqueous solvents is by no means a new technique. As early as 1903 Vorländer (85), using indicators, observed color changes with gaseous hydrogen chloride. Probably the first titration in nonaqueous medium was performed by Folin, Flanders, and Wentworth (18–20) in 1910, when various fatty acids were dissolved in organic solvents and titrated with sodium ethylate. Fundamental work on acid-base reactions in glacial acetic acid was carried out by Conant, Hall, and Werner in 1927 (7, 8, 30–32). Kolthoff and Willman (48, 49) in 1934 investigated the strength of cations and anions in glacial acetic acid, while in the same year Nadeau and Branchen (59) published their method for the determination of amino acids in acetic acid. Blumrich and Bandel (4) determined several organic bases in glacial acetic acid using perchloric acid as titrant.

Since 1950 a number of strong protagonists emerged, who have contributed considerably to this rapidly expanding field and have demonstrated the importance of nonaqueous titrations to pharmaceutical analysis. Among these are Markunas and Riddick (55) who investigated a great number of organic compounds for their suitability to titrations in glacial acetic acid and who reported accuracy and precision obtainable. A review of nonaqueous titrations has appeared in *Analytical Chemistry* (71). Davis, *et al.* (11–13), at the National Bureau of Standards studied indicators and electrode systems in various organic solvents. The application of nonaqueous titrations to acidic and basic compounds has been furthered greatly by Fritz (21). Several new techniques have been developed by the authors such as the use of mercuric acetate for the direct titration of halide salts of organic bases (63, 64), the semiautomatic extraction followed by direct titration of the organic acid or base in the solvent (72), and the study of the

* Received May 26, 1953, from the Products Control Laboratory, Hoffmann-La Roche, Inc., Nutley, New Jersey.
Presented to the Eastern and Central Scientific Sections of the American Pharmaceutical Manufacturers' Association in New York on February 3, 1953, and in Chicago on February 9, 1953, under the title "Titrations in Nonaqueous Solutions."
The authors are indebted to E. G. E. Shafer for his valuable contributions to this paper.

effects of solvents for increasing the sensitivity of titrations in nonaqueous solutions (66). Higuchi and his coworkers determined the active hydrogen of various organic compounds by titration with lithium aluminum hydride or amide in tetrahydrofuran (40, 53), while Tomiček, Doleĉal, and Heyrovsky studied redox titrations and the bromination of phenolic compounds (78, 79). A number of surveys have appeared in the literature, covering various aspects of titrations in nonaqueous solutions, especially as applied to pharmaceuticals (2, 3, 15, 46, 54, 79, 80).

The importance of titrations in nonaqueous solutions to the pharmaceutical field can be attributed to the following qualifications:

Specificity.—Depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant.

Solubility.—The variety of organic solvents available permits the choice of the most desirable for solubilizing the sample.

Simplicity.—Most of the titrations involved are of an acid-base nature, which can be performed either visually using a variety of indicators, or potentiometrically with various electrode combinations. These methods are advantageous for routine control, since a minimum of equipment is required, the determinations can be carried out rapidly, and the solvents and indicators used are available at moderate cost.

Sensitivity.—Organic solvents permit far less ionization than an aqueous medium. By proper selection of solvents, therefore, many determinations can be conducted with very small quantities of sample without loss of sensitivity at the end points.

Selectivity.—Recently developed techniques permit the differentiation between various acidic or basic functional groups within the same molecule or in mixtures—a procedure which usually cannot be carried out in aqueous solution.

Accuracy and Precision.—The accuracy and precision obtainable is comparable in most cases to those of conventional titrations in water.

THEORY

The purpose of this paper is to present the practical applications of nonaqueous titrations to pharmaceutical compounds and their intermediates. No attempt is made to delve into the theoretical aspects, since these have been covered thoroughly in previous publications. An excellent review and bibliography of the theoretical considerations can be found in Riddick's paper, "Acid-Base Titrations in Nonaqueous Solvents" (71). Other good sources are, *Advanced Organic Chemistry* by Wheland (87), *Acidimetry in Organic Solvents* by Wolff (88) and *Acid-Base Titrations in Nonaqueous Solvents* by Fritz (21). A recent book, *Non-Aqueous Solvents* by Audrieth and Kleinberg has been published by John Wiley & Sons, Inc., New York, 1953.

TITRANTS

Acidic Titrants

In most titrations it is desirable to use the most reactive titrant available. Perchloric acid fills this requirement and is widely used as the acid titrant. Kolthoff and Willman (48, 49) determined the dissociation constants of some inorganic acids in glacial acetic acid and listed the strongest ones in the following order of decreasing acidity:

$$HClO_4 > HBr > H_2SO_4 > HCl > HNO_3$$

Perchloric Acid (0.1 *N*) in Acetic Acid.—This is prepared by diluting 8.4 cc. 70% perchloric acid to 1,000 cc. with glacial acetic acid. Several investigators have proposed the addition of acetic anhydride to the above solution to eliminate the small quantity of water introduced by the 70% perchloric acid. It should be noted that acetic anhydride should never be poured directly into perchloric acid, but only after a large quantity of glacial acetic acid has been added.

Perchloric acid (0.1 *N*) in *p*-dioxane may be used in cases where a glacial acetic acid solution of perchloric acid is not a desirable titrant. This system offers several advantages. It tends to increase the reactivity of the titrant by decreasing its ionization, and it permits the direct titration of basic compounds dissolved in either glacial acetic acid or in a neutral solvent using the same titrant. A solution of 0.1 *N* $HClO_4$ is prepared by diluting 8.4 cc. 70% perchloric acid to 1,000 cc. with purified *p*-dioxane. *p*-Dioxane, as obtained commercially, is often impure and will discolor when perchloric acid is added. It can be purified by distillation over sodium, adsorption columns, or by mixing with Amberlite IRA 400 (OH) resin (Rohm and Haas Co.) followed by filtration. A recent very efficient and simple purification procedure is described by Sideri and Osol in THIS JOURNAL, who shook *p*-dioxane with asbestos and filtered the solvent.

Hydrobromic Acid (0.1 *N*).—This has been prepared in a similar manner for the analysis of epoxy compounds.

Strong Organic Acids.—Several of these have recently been investigated by Smith and Elliott (77), who measured their acid strength and dissociation constant in glacial acetic acid, listing those as stronger than perchloric acid in the following order of decreasing acidity: methanetrisulfonic acid > chloromethionic > methionic > perchloric acid. The practical applications of these acids to nonaqueous titrations remain to be explored.

Basic Titrants

The metallic alcoholates are primarily used as the basic titrant (21).

Lithium Methoxide (0.1 *N*).—Some disadvantages have been encountered in our laboratory when using the more common alkali metal alcoholates such as sodium or potassium methoxide in benzene-methanol solution; these alcoholates often produce gelatinous precipitates with organic acids, which may obscure the visual end point or coat the electrodes, causing a sluggish inflection. The authors therefore recommend the use of lithium methoxide in benzene-methanol, since we rarely have encountered this undesirable condition.

Lithium methoxide (0.1 *N*) is prepared by dissolving approximately 0.6 Gm. of freshly cut lithium metal in 150 cc. of absolute methanol, while cooling the flask in ice water. When the reaction is completed, 850 cc. of dry benzene is added. The alcohol should be kept to a minimum. If cloudiness or precipitation of the lithium methoxide occurs, enough additional methanol is added to clear the solution. The reagent is stored in the reservoir of an automatic delivering burette protected from carbon dioxide and moisture.

Sodium Methoxide (0.1 *N*).—This is prepared by dissolving 2.3 Gm. of freshly cut sodium metal in about 100 cc. of absolute methanol, while cooling the flask in ice water. When the reaction is completed, 50 cc. additional methanol is added, followed by 850 cc. of dry benzene. The alcohol concentration should be kept to a minimum. If cloudiness or precipitation of sodium methoxide occurs, enough additional methanol is added to clear the solution. Storage similar to lithium methoxide.

Potassium Methoxide (0.1 *N*).—Since a base becomes the strongest base in the solution in which it is least ionized (33, 34), the smallest possible amount of alcohol should be used as solvent as it tends to increase the ionization. About 150 cc. absolute methanol is required per 850 cc. benzene to hold 0.1 *N* sodium methoxide or lithium methoxide in solution. Under the same conditions, when 0.1 *N* potassium methoxide is prepared, only about 75 cc. of absolute methanol per 925 cc. benzene is necessary. Recent unpublished work carried out in this laboratory has shown that potassium methoxide is a stronger base than sodium or lithium methoxide and therefore may be advantageous in certain instances where a more powerful titrant is desired.

Potassium methoxide (0.1 *N*) is prepared by dissolving approximately 4 Gm. of freshly cut potassium metal in a solution of about 20 cc. absolute methanol and 50 cc. dry benzene, while cooling the flask in ice water. When the reaction is completed, 55 cc. of absolute methanol are added and the solution diluted to 1 L. with dry benzene. The alcohol should be kept at a minimum. If cloudiness or precipitation of potassium methoxide occurs, enough additional methanol is added to clear the solution. Storage similar to lithium methoxide.

Sodium Aminoethoxide.—This is used for potentiometric titration of weakly acidic phenolic compounds. Moss, Elliott, and Hall (58) proposed the use of ethylenediamine as solvent and sodium aminoethoxide as titrant and obtained excellent results in titrating dark colored resin mixtures.

Sodium aminoethoxide (0.1 *N*) (44) is prepared by dissolving approximately 5.0 Gm. of freshly cut sodium metal in 200 cc. freshly purified ethanolamine with cooling. The resulting solution is diluted to 1,000 cc. with ethylenediamine.

Sodium Triphenylmethane.—This is an extremely strong base and is used for the titration of very weakly acidic compounds such as pyrroles. It is prepared in 1:1 benzene-ether solution. The deep red color of the solution serves as its own indicator.

Sodium triphenylmethane (0.1 *N*) is prepared in a special apparatus (9) by dissolving approximately 20 Gm. (9–10 cc.) of 45% sodium amalgam in 100 cc. of dry ether. A solution of 12 Gm. of pure triphenyl methylchloride in 100 cc. benzene is placed in the separatory funnel. The flask is evacuated and filled three or four times with nitrogen to replace the air. The benzene solution is run into the flask below, which is shaken for twelve to fourteen hours after all the reagent has been added. The sodium triphenylmethane is filtered from the sodium chloride and mercury through a sintered glass funnel into a storage vessel. A mixture of 125 cc. of dry ether and 125 cc. of dry benzene is added from an ether separatory funnel. The resulting solution has a normality of about 0.07 to 0.08 and can be kept for weeks with very little change in strength.

Reducing Titrants

Lithium Aluminum Hydride and Amide.—Lintner, Schleif, and Higuchi (52) have used lithium aluminum hydride and lithium aluminum amide for the titration of easily reducible compounds such as glycols, alcohols, aldehydes, and esters. An excess of titrant in tetrahydrofuran is added, allowed to react, and the excess of lithium aluminum hydride backtitrated with a standard solution of *n*-propanol in benzene.

Lithium aluminum hydride (1 *N*) is prepared by placing about 25 Gm. of lithium aluminum hydride in 1 L. of tetrahydrofuran and refluxing for 24 hours. The supernatant liquid is removed and kept under nitrogen. An automatic burette is used for the titration.

Lithium aluminum amide (1 *N*) is obtained by adding a slight excess of dry di-*n*-butylamine or piperidine to 1 L. of lithium aluminum hydride solution, prepared as above, and the solution stored under nitrogen.

STANDARDIZATION OF TITRANTS

Standardization of Acidic Titrants

Perchloric acid can be used as titrant in glacial acetic acid or in a neutral organic solvent. In the glacial acetic acid solvent system the preferred primary standard is potassium acid phthalate, as proposed by Seaman and Allen (74). The standardization can be carried out either visually or potentiometrically, depending upon the procedure chosen for the titration of the sample.

Perchloric Acid (0.1 *N*) in Glacial Acetic Acid.—This is standardized against approximately 0.8 Gm. of potassium acid phthalate N. B. S. (National Bureau of Standards), dried to constant weight, and accurately weighed into a 250-cc. beaker or Erlenmeyer flask. Eighty cc. of glacial acetic acid are added and the contents heated to effect complete solution. Approximately 5 drops of crystal violet, methyl violet, or α-naphtholbenzein (1% in glacial acetic acid) are added and the solution is titrated with the perchloric acid solution, preferably with

mechanical stirring. The standardization may also be conducted potentiometrically, using a glass-calomel electrode system. In visual titrations the sample should be titrated to the same color shade as the standard. Since visual and potentiometric titrations will not necessarily result in identical standardization factors, the titration of the sample should be carried out in the same manner as the standardization.

Perchloric Acid (0.1 *N*) in Neutral Solvents.—Potassium acid phthalate is insoluble in neutral organic solvents. In such instances diphenylguanidine (22, 23) can be used as primary standard. Diphenylguanidine (Eastman) is recrystallized (6) from 95% ethanol and then from toluene, followed by drying to constant weight at 110°. Approximately 0.7 Gm. is weighed accurately into a 250-cc. Erlenmeyer flask. It is dissolved in about 20 cc. *p*-dioxane and diluted with 80 cc. benzene. Five drops of methyl red indicator (1% in absolute ethanol) are added and the sample titrated to a red end point with perchloric acid. In this case it is essential to use a titrant consisting of perchloric acid in *p*-dioxane as described under "Titrants" above. The visual and potentiometric end point will result in the same standardization factor and therefore one may be substituted for the other.

Standardization of Basic Titrants

All basic titrants, including potassium, sodium, and lithium methoxide, sodium aminoethoxide, and sodium triphenylmethane are best standardized against benzoic acid (N. B. S.).

0.1 *N* Basic Titrants.—Approximately 0.4 Gm. benzoic acid N. B. S. is accurately weighed and dissolved in 80 cc. of the basic solvent of choice, as described under "Solvents" below. The titration is performed visually, using 5 drops of a 1% solution of thymol blue, α-naphtholbenzein, *p*-nitrobenzeneazoresorcinol, or other suitable indicator (see Table II) in a basic solvent, or potentiometrically using electrodes (see Table III).

Standardization of Reducing Titrants

1 *N* Lithium Aluminum Hydride or Amide (41).—Reagent grade *n*-propanol is distilled over sodium, collecting the middle fraction. Fifty Gm. of this purified *n*-propanol is diluted to 1 L. with dry thiophene-free benzene and stored in the reservoir of an automatic burette. Lithium aluminum hydride or lithium aluminum amide is standardized against this solution by use of *n*-phenyl-*p*-aminoazobenzene as indicator, or by a potentiometric method (see Table III).

SOLVENTS

The proper selection of the solvent system may contribute to the success of many a titration. The dielectric constant of the solvent has a direct effect upon the dissociation of the compound. According to Hammett (33, 34), "any specified acid forms the most acidic solution—i. e., the solution which is the most effective proton donor—in that solvent in which it is least ionized." The same concept applies to basic compounds. In order to obtain a solution in which the ionization is depressed, it is often necessary to dissolve the sample in a minimum quantity of acid or basic solvent to effect solubilization, followed by an excess of a miscible organic solvent of low dielectric constant, e. g., benzene to acidic or basic solvents, or *p*-dioxane to water. This technique increases the sharpness of the visual and potentiometric end points and is particularly useful when applied to titrants of 0.02 *N* or lower normality. It has been proved that the ionization of a compound is not a requirement for a titration since those carried out in aprotic solvents are far more sensitive (66).

Riddick (55) has set forth some general requirements for solvents such as: (*a*) they must be commercially available in reasonably pure form at moderate cost; (*b*) they must dissolve the substance being titrated; (*c*) the products of the titration should be free from gelatinous-type precipitates; and (*d*) the solvents should not enter into the reactions. Solvents listed below will meet these requirements in many cases. They can be conveniently classified into four groups: acidic, basic, relatively neutral, and mixed solvents.

TABLE I.—RELATIVELY NEUTRAL SOLVENTS

Acetonitrile	c. p. grade
Benzene	c. p. grade
Chlorobenzene	c. p. grade
Chloroform	c. p. grade
p-Dioxane	c. p. grade
Ethylacetate	c. p. grade
Methanol (absolute)	c. p. grade
Nitrobenzene	c. p. grade
Toluene	c. p. grade
Tetrahydrofuran	Technical grade (Du Pont)[a]
Xylene	c. p. grade

[a] This is distilled twice over lithium aluminum hydride. This solvent should be purified regularly since it may contain peroxides.

Acidic Solvents

Glacial Acetic Acid.—This acid possesses excellent properties for solubilizing most organic compounds. It is readily available and may be used in either reagent grade or technical grade, providing a blank is first run. Small quantities of water may be eliminated by heating after addition of acetic anhydride (except for the titration of primary and secondary amines).

While other organic acids have been used, thus far glacial acetic acid seems to be the preferred solvent.

Basic Solvents

Dimethylformamide (D. M. F.).—This is commercially available from the E. I. Du Pont Co. and requires no additional purification. It possesses little odor and has been successfully used for the titration of many organic acids with the exception of phenols.

***n*-Butylamine.**—This is a good solvent requiring no further purification when used for the titration of weak organic acids. It absorbs carbon dioxide more readily than D. M. F. and has somewhat of a disagreeable odor.

Pyridine.—It is a good solvent except for its disagreeable odor.

TABLE II.—INDICATORS

Solvent System	Indicator	Reference[a]
Acidic Solvents:		
Glacial acetic acid	Crystal violet, Methyl violet, α-Naphtholbenzein	(59, 74, 23)
	Bromophthalein Magenta E	(11, 12, 13)
	Metanyl yellow, Neutral red	(80)
	Benzylauramine	(59)
	Blue BZL (Ciba, Basle, catalog No. 22062S)	(16)
Relatively Neutral Solvents:		
Chloroform, p-Dioxane, Benzene, Alcohols, Chlorobenzene, Acetonitrile	Methyl red, Modified methyl orange	(22)
Tetrahydrofuran	N-Methyl-p-aminoazobenzene, N-Phenyl-p-aminoazobenzene	(41)
Glycol-Isopropanol	Methyl red	(62)
Basic Solvents:	Thymol Blue	(27, 29, 25)
Dimethylformamide, Pyridine	α-Naphtholbenzein	(25, 27, 29)
	p-Nitrobenzeneazoresorcinol (azo violet)	(27, 29)
n-Butylamine	o-Nitroaniline	(28)
Ethylenediamine	p-Hydroxyazobenzene	(28)

[a] For special reactions of acids and bases with indicators in aprotic solvents see reference (70). For fluorescence indicators in glacial acetic acid see reference (81).

Ethylenediamine.—Anhydrous ethylenediamine in a pure form of 95 to 100% is desirable. Moss, *et al.* (58), recommend the removal of all water. It is an excellent solvent for the titration of phenols and very weakly acidic organic acids. It can be purified by distillation over sodium as described by Putman and Kolbe (69).

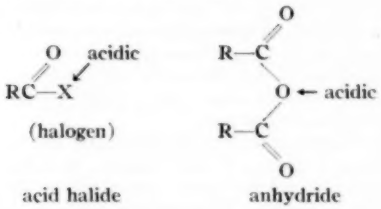
Mixed Solvents

Relatively neutral solvents of low dielectric constant, such as benzene, toluene, *p*-dioxane or chloroform, may be used in conjunction with any of the acidic or basic solvents in order to increase the sensitivity of the titration end points (66).

APPLICATIONS

Acidic Functional Groups

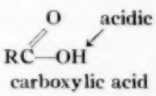
Acid halides and anhydrides.—



acid halide anhydride

Acid halides and anhydrides are often encountered as intermediates in the manufacture of pharmaceutical compounds. They usually can be dissolved in dimethylformamide (D. M. F.), an excess of titrant added, heated if necessary, and the excess back-titrated with perchloric acid in *p*-dioxane using thymol blue as indicator.

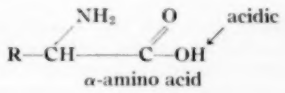
Carboxylic acids and amino acids.—



carboxylic acid

The acidity of this functional group is sufficiently strong to allow titration with aqueous sodium hydroxide. If these acids are water-insoluble, they can be dissolved in a basic solvent and titrated with one of the basic titrants (e. g., 0.1 *N* sodium methoxide using thymol blue as indicator).

Examples: benzoic acid, acetylsalicylic acid, *p*-aminobenzoic acid, and stearic acid.

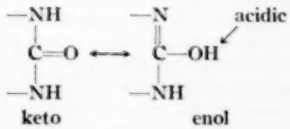


α-amino acid

This compound contains a basic and an acidic moiety. The titration of the basic amine is described under "Bases." The acidic carboxyl group may be titrated as described above.

Examples: glycine, alanine, and methionine.

Enols.—



keto enol

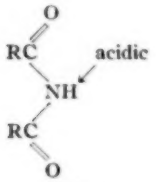
Good examples of acidic enolic compounds are the barbiturates (25, 84) which can be titrated in

D. M. F. with 0.1 *N* sodium methoxide, using thymol blue or azo violet as indicator.

Examples: amobarbital, barbital, pentobarbital, phenobarbital, and seconal.

The sodium salts of these barbiturates can be determined upon liberation of the free barbituric acid with sulfuric acid, followed by extraction with an organic solvent. About 10 cc. of D. M. F. is added to the organic solvent and the enolic compound titrated with 0.1 *N* sodium methoxide using thymol blue as indicator.

Imides.—

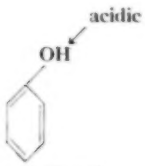


imide

Fritz (25) titrated imides in D. M. F. using azo violet or thymol blue as indicator and suggested for very weakly acidic imides and enols that they be titrated in ethylenediamine with *o*-nitroaniline indicator. Fritz showed that imides containing the thiocarbonyl group are stronger acids than those having a carbonyl group. Theobromine has been titrated in ethylenediamine with azo violet as indicator.

Examples: saccharin, phthalimide, succinimide, and theobromine.

Phenols.—



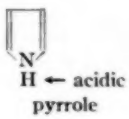
phenol

Moss, Elliott, and Hall (58) suggested the use of ethylenediamine as solvent for the titration of weakly acidic phenolic compounds. The success of the method depended upon the absence of water in the solvent and the end point was obtained potentiometrically using sodium aminoethoxide as titrant. Ethylenediamine can be made anhydrous by distillation over sodium as described by Putman and Kolbe (69).

In a recent paper, Fritz and Keen (28) described visual titrations of various phenols in either dimethylformamide or ethylenediamine, depending upon the acidity of the phenol being titrated. These authors proposed azo violet as indicator for the determination of phenols in D. M. F. if the aromatic phenol had an aldehyde, keto, ester, nitro, or amide group in the *ortho*- or *para*-position. The above substituted phenols are more acidic than the unsubstituted phenols and naphthols, which should be titrated in ethylenediamine using *o*-nitroaniline as indicator and potassium methoxide in benzene-methanol as titrant. Carboxylic acids and phenols can be differentially titrated in the presence of each other with acetone or acetonitrile as a solvent. Using *p*-hydroxyazobenzene as the indicator, only the carboxylic acid will titrate, while in certain cases the addition of azo violet indicator at this point will permit the continued titration of the phenolic content to a blue end point. Further possible modifications of this technique are described in the above mentioned paper.

Examples: *p*-hydroxybenzaldehyde, 8-hydroxyquinoline, methyl salicylate, β-naphthol, phenol, salicylamide, and vanillin.

Pyrroles.—



pyrrole

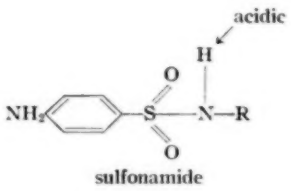
These compounds are very weakly acidic and have been titrated with sodium triphenylmethane (9), which serves as its own indicator.

Salts and Acid Gases.—The acidic moiety of an organic or inorganic salt (24) can in many instances be titrated in nonaqueous solvents. A variety of solvents such as dimethylformamide, ethylenediamine, and morpholine were used with sodium methoxide as titrant and azo violet or thymol blue as indicator.

Examples: caffeine sulfate, quinine sulfate, morphine hydrochloride, and codeine phosphate.

Acid gases such as CO_2, SO_2, H_2S, etc., when absorbed in a basic solvent, may be titrated with sodium methoxide.

Sulfonamides.—



sulfonamide

The sulfonamides have amphoteric character, with a basic primary aromatic amine in addition to an acidic hydrogen of the monosubstituted sulfonamide. Most sulfonamides may be titrated in D. M. F. using thymol blue as indicator.

Examples: sulfanilamide, sulfadiazine, sulfapyridine, sulfamerazine, succinylsulfathiazole, and sulfisoxazole (Gantrisin[1]).

The degree of acidity depends upon the influence of the R radical. If the R-group is aromatic, it decreases the acidity of the compound and therefore requires a stronger basic solvent such as *n*-butylamine.

Fritz and Keen (27) prepared several substituted sulfonamides which do not contain the primary aromatic amine group and therefore cannot be titrated by the U. S. P. sodium nitrite method.

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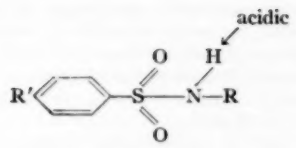
[1] Trade mark Reg. U. S. Pat. Off.

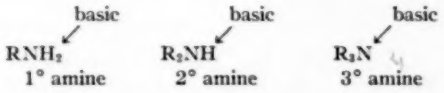
Example:



Tomiček (77*a*) titrated the primary aromatic amine of a number of sulfonamides in acetic acid with perchloric acid.

Basic Functional Groups

Amines.—



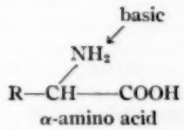
Primary, secondary, and tertiary amines can be titrated in any of the relatively neutral solvents with perchloric acid in *p*-dioxane using methyl red as indicator. They also can be dissolved in glacial acetic acid and titrated with perchloric acid in either acetic acid or *p*-dioxane, with crystal violet as indicator. The technique of microtitration of amines has been demonstrated by Fritz who performed many titrations on very dilute solutions (45).

Examples: histamine, metamphetamine, ethanolamines, ethylamines, amphetamine, and epinephrine.

Mixed aliphatic amines (86) and mixed aromatic amines (76), can be determined by the following technique. The mixture is dissolved in glacial acetic acid and brought to a known volume. An aliquot is titrated with perchloric acid to give the total basic amine content (*A*). To another aliquot acetic anhydride is added and the solution heated to boiling in order to acetylate the primary and secondary amines. This solution is likewise titrated to indicate the basicity due to the tertiary amine (*B*). The difference (*A* − *B*) will provide a measure of the basicity (*C*) due to the primary and secondary amines. Salicylaldehyde in acetic acid is added to a third aliquot which upon warming forms a Schiff's base, having only very weakly basic properties. This solution is then titrated to measure the basicity due to the secondary amine (*D*). Therefore (*C* − *D*) represents the basicity due to the primary amine.

Differential potentiometric titrations of aliphatic, aromatic, and heterocyclic amines have been accomplished by Fritz (26), using acetonitrile as solvent. He also compared the basic strength of a number of amines in water and acetonitrile. The authors (67) have carried out a similar differentiation including inorganic cations in a chloroform-acetic acid mixture.

Amino Acids.—



The basicity of amino acids may be titrated by dissolving the sample in glacial acetic acid, adding a measured excess of perchloric acid, and back-titrating with either sodium acetate in acetic acid or sodium methoxide in benzene-methanol, using crystal violet as indicator (59). Since the carboxyl group in amphoteric amino acids exhibits a relatively strong acidic property, the direct titration of the acidity is usually preferred.

Examples: glycine, leucine, and histidine.

TABLE III.—ELECTRODES

Solvent System	Electrodes	Reference
Acidic Solvents:		
Glacial acetic acid	Glass calomel[a]	
	Antimony-tellurium	
	Hydrogen electrode	(78, 80)
	Antimony-calomel	
	Metal electrodes (others)	
	Silver-silver chloride	(23)
Relatively Neutral Solvents:		
Acetonitrile		(26)
Chloroform	Glass-calomel	(65)
Alcohol		(66)
Tetrahydrofuran	Platinum-calomel	(37, 38)
Ethyl acetate		
Nitrobenzene	Glass-silver-silver chloride	(23)
Chlorobenzene		
Basic Solvents:		
Dimethylformamide	Antimony-calomel	(21)
n-Butylamine	Antimony-glass	(29)
Pyridine	Antimony-glass	(29)
Ethylenediamine	Antimony-antimony	
	Hydrogen-antimony	(58)
	Hydrogen-calomel	
Oxidation-Reduction:	Platinum-calomel	(78)
Additional Electrode Systems:		see (21)

[a] The commonly used electrode system.

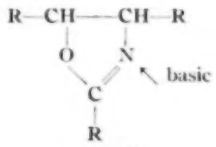
Heterocyclic Nitrogen Compounds.—

heterocyclic nitrogen compound

These compounds may be titrated with perchloric acid in *p*-dioxane in relatively neutral solvents using methylred as indicator, or in glacial acetic acid with crystal violet indicator.

Examples: niacinamide (64), isoniazid (1), aminopyrine (51), theobromine (68), quinine (35), cocaine (64), strychnine (35), purine bases (14), and pyrazolones (83).

Oxazolines.—

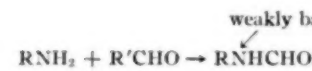


oxazoline

Certain oxazolines (55) may be titrated directly in acetic acid while others require heating with a measured excess of 0.1 *N* perchloric acid, followed by backtitration with 0.1 *N* sodium methoxide. No other method of analysis for oxazolines has been reported.

Example: 2-nonyl-4,4-bis(hydroxymethyl)-2-oxazoline.

Schiff Bases.—



Schiff base

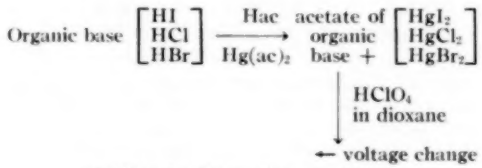
TABLE IV.—EQUIVALENTS CONSUMED BY FUNCTIONAL GROUPS OF REDUCIBLE COMPOUNDS

Reducible Functional Group	Moles Compound	Moles Reagent ($LiAlH_4$)
Alcohols, glycols, phenols	4	1
Carboxylic acids	4	3
Primary amines	2	1
Secondary amines	4	1
Aldehydes	4	1
Ketones	4	1
Esters	2	1
Anhydrides	1	1
Amides, disubstituted	4	2
Amides, monosubstituted	4	3
Nitriles	2	1
Aromatic nitro group	2	2
Water	4	1
Ammonia	5	2
Metal halides	4	7
Examples: Salicylic acid	Benzaldehyde	
Amidopyrine	Vanillin	
Coumarine	Acetylsalicylic acid	
Morphine		
Cinchonidine	Phenyl salicylate	
Methyl Salicylate	Thymol	
	Menthol	

Schiff bases are formed by the reaction of a primary amine with an aldehyde to produce a very weakly basic compound. A number of these have been titrated potentiometrically in chloroform or glacial acetic acid with perchloric acid by S. Freeman of the Benzol Products Corporation in Newark, New Jersey. This work will be published in *Analytical Chemistry*.

Salts.—Salts of amines, basic heterocyclic nitrogen-, quaternary ammonium-, and inorganic compounds.

Organic Salts, composed of basic organic or inorganic groups and acids other than the halogen acids, can be titrated directly in acetic acid with perchloric acid using visual indicators or potentiometrically. Halogen acid salts of such bases are acidic in glacial acetic acid and therefore can be titrated only after the addition of neutral mercuric acetate (63, 64).



perchlorate of organic base + Hac

Procedure.—Approximately 3 milliequivalents of the salt is weighed into a 150-cc. beaker and dissolved in 80 cc. glacial acetic acid with heating, if necessary. In the case of the halogen acid salts 15 cc. of a 6% solution of mercuric acetate in glacial acetic acid is added, followed by approximately 5 drops of crystal violet indicator. The titration is performed with 0.1 *N* perchloric acid in *p*-dioxane, matching the color shade of the end point with the one obtained in the standardization of the perchloric acid solution. The titration also can be carried out potentiometrically using a glass-calomel electrode system.

Sulfuric acid salts of organic bases (64), if titrated directly, will consume only one equivalent instead of the expected two; since in the reaction an acid sulfate is formed which is strongly acidic in glacial acetic acid. Mercuric acetate will not eliminate the effect of the acidic bisulfate ion. Mercurous acetate, however, when added to a solution of the sulfate salt in acetic acid (heating required), removes the sulfate ion completely and permits the titration of both basic equivalents.

Example: Amine Salts:
ephedrine HCl
histidine HCl
atropine sulfate
amphetamine sulfate
Heterocyclic Nitrogen Salts:
papaverine HCl
codeine HCl
morphine HCl
caffeine sulfate
thiamine HCl
pyridoxine HCl
phenindamine (Thephorin[2])

[2] Trade mark Reg. U. S. Pat. Off.

Quaternary Ammonium Salts:
choline chloride (10, 56)
neostigmine bromide (Prostigmin[3] bromide)
carbamylcholine chloride (carbachol)
alkyldimethylbenzylammonium chloride (benzalkonium chloride)
Antihistamine Salts (47)
Narcotic Salts (51)
Pyridine Salts (43)
Antibiotics (16)

Inorganic Salts.—Many inorganic salts have been titrated as bases with perchloric acid in *p*-dioxane (65). A number of these salts are rather difficult to solubilize in glacial acetic acid and it may be necessary to pulverize the sample to a 200-mesh particle size and reflux it with glacial acetic acid to effect solubilization. When a solution cannot be achieved in this manner, the salt may be dissolved in no more than 5 cc. water, followed by the addition of 100 cc. glacial acetic acid. Titrations of inorganic salts must be carried out potentiometrically if water is present.

Anions, listed as follows, have been titrated as their sodium salts. (Mercuric acetate must be added to halogen salts.)

Anions:

acetate	chloride	nitrate
azide	cyanide	peroxide
bicarbonate	fluoride	phosphate
bisulfite	hydroxide	silicate
borate	hydrophosphite	sulfate
bromate	iodate	sulfide
bromide	iodide	sulfite
carbonate	molybdate	thiocyanate
chlorate	nitrite	tungstate

Salts composed of the cations, listed in the following table, with any of the anions mentioned above also may be titrated in the manner described, providing they can be solubilized and react basic in glacial acetic acid.

Cations:

aluminum	cadmium	manganese
ammonium	calcium	potassium
antimony	cobaltous	silver
barium	iron	strontium
bismuth	lead	zinc
	magnesium	

Higuchi, *et al.* (37, 38), also reported on the titration of inorganic salts in glacial acetic acid. Lithium chloride and lithium bromide have been titrated in glacial acetic acid with mercuric acetate, lead acetate, or tellurium acetate either visually or potentiometrically (82).

Reducible Functional Groups

The potentiality of lithium aluminum hydride as a reducing agent was recognized by Zaugg and Horrom (89), Krynitsky, *et al.* (50), and Hochstein (42), who utilized gasometric measurements of the liberated hydrogen for analytical purposes.

Higuchi, *et al.* (36, 39, 40, 41, 52, 53), and Olleman (61) have applied both lithium aluminum hydride and lithium aluminum amides to the volumetric analysis of a variety of easily reducible compounds, many of which are of interest to the pharmaceutical industry. The methods entail essentially the addition of a measured excess of the powerful reducing agent, allowing the reaction to come to completion while standing for a period of time, and back-titration with standardized *n*-propanol solution in benzene (52). The sharp end point is determined potentiometrically with silver-saltbridge-silver electrodes or visually using *N*-methyl-*p*-aminoazobenzene or preferably *N*-phenyl-*p*-aminoazobenzene as indicator. The titration is performed in a closed system under nitrogen, since atmospheric oxygen and moisture interfere (36). For titrants, standardization, solvents, indicators, electrodes, see *loc. cit.* Table IV, p. 516, lists the number of equivalents consumed by various functional groups of reducible compounds when reacted with lithium aluminum hydride (53).

[3] Trade mark, Reg. U. S. Pat. Off.

Brominations

Tomiček and Doležal (78) have shown that saturated organic compounds can frequently be brominated in acetic acid by substitution, while unsaturated compounds react with bromine by addition. The titrations are performed potentiometrically with 0.1 *N* Bromine in glacial acetic acid in the presence of sodium acetate. The extent to which the bromination proceeds, depends often upon the temperature chosen and the presence or absence of sodium acetate. Similar work has been reported by Braae (5) who titrated unsaturated hydrocarbons with bromine in carbon tetrachloride.

Examples: phenol, pyrocatechol, hydroquinone, resorcinol, thymol, cholesterol, limonene, and oleic acid.

Redox Titrations

Tomiček and Heyrovsky (80) investigated the possibility of oxidation and reduction titrations in glacial acetic acid. These authors used as oxidizing titrants chromic acid, sodium permanganate, or bromine, all dissolved in glacial acetic acid. The potentiometric titrations were carried out on a semimicro scale with a calomel-hydrogen electrode system (a platinum electrode coated with palladium black). Reductions were performed with titanous chloride in glacial acetic acid. Among the inorganic substances oxidized were arsenic, antimony, mercury, iron, chromium, bromides, and iodides. Organic compounds titrated were dihydric phenols, aminophenols, and diphenylamine.

Karl Fischer Titrations

The determination of water by means of the Karl Fischer reagent (17) must necessarily be included in any survey of nonaqueous titration methods. This extremely useful procedure is not only applicable to the determination of superficial moisture and water of hydration of organic or inorganic compounds, but also to organic reactions involving the liberation or consumption of water. Thus it can be utilized for the determination of functional groups such as alcoholic hydroxyl, carboxylic acids, acid anhydrides, carbonyl, amines, nitriles, peroxides, etc. A comprehensive treatise on this subject can be found in Mitchell and Smith's book *Aquametry* (57).

Pharmaceutical Preparations

Tablets, Capsules, and Powders.—Tablets, capsules, and powders containing no interfering substances can be assayed by direct titration of the pulverized mass.

When assaying acidic compounds in the presence of excipients, such as stearic acid, erroneously high values will be obtained, unless the interfering substance is first removed. In the case of stearic acid this can often be accomplished by extraction with petroleum ether.

In the assay of basic compounds or their salts, interferences may be encountered from inert ingredients such as magnesium stearate, calcium, or sodium salts. If the active ingredient to be determined is the halogen acid salt of an organic base, the interfering basic excipient often may be eliminated in the following manner (64). The pulverized tablet mass, after heating with glacial acetic acid, is titrated with perchloric acid to the indicator end point (titration of the basic excipient). Mercuric acetate solution is then added and the titration continued to the renewed appearance of the end point (titration of the total basicity). The volume difference between the first and second titration constitutes the milliliter perchloric acid consumed by the active ingredient.

Another very useful technique (72) is to extract the acidic or basic active ingredient with an organic solvent as described under "Extraction Techniques," below.

Solutions (Including Parenteral Solutions and Syrups).—In the absence of interfering ingredients such as buffers, certain preservatives, etc., a measured quantity of the solution can often be evaporated to dryness on a steam bath, the residue dissolved in the proper solvent, and titrated. In other instances the active ingredient must be extracted.

Ointments.—The same technique as described under "Solutions" is applicable to ointments. In the case of ointment bases composed only of polyethylene glycols (Carbowaxes) the titration of the active ingredient is performed directly in the absence of appreciable quantities of water.

Extraction Techniques

In order to titrate the active ingredient in many pharmaceutical preparations, it is necessary to isolate it from interfering excipients and carriers. This can be accomplished by extraction from the solid powdered tablet mass or by means of liquid-liquid extractors. The extracted compound is titrated directly in a nonaqueous solvent.

Extraction of Solid Materials.—The best-known type of apparatus used for extraction from the solid phase is the Soxhlet extractor, which requires refluxing of the solvent. This method is applicable only if the compound is soluble in the extracting solvent and will not decompose on prolonged heating. In those cases where the extracted compound has acidic or basic properties, it can be titrated directly in the organic solvent (best solvents: benzene, chloroform, toluene, xylene, etc.). Ether and alcohols must be evaporated and the residue dissolved in one of the solvents best suited for the titration.

Another simple apparatus for the extraction of solids with organic solvents, but without the use of heat, is described by Napoli and Schmall (60). This apparatus consists of a glass-stoppered dropping funnel with a sintered glass plate to separate the solid and liquid phase. The liquid phase can be titrated in a similar manner as described for the Soxhlet extractor.

Extraction by Immiscible Solvents.—*Squibb Separators.*—This technique is usually applied for the determination of organic acids, bases, and their salts in preparations such as liquids, ointments, tablets, capsules, etc. The Squibb separator is the most commonly used tool for the separation of immiscible solvent phases. By applying sufficient extractions and combining the organic solvent phases, it is possible to titrate the extracted compound directly. However, care must be taken to assure a clean-cut separation of the phases and to prevent any of the aqueous layer from being carried over into the combined organic solvent phase.

Schmall Extractors.—The recently developed "Schmall" extractors (72) are better applicable to nonaqueous methods of analysis than the Squibb separators. They permit semiautomatic extraction with a clean separation of the solvent phase, thus assuring sharper end points, since no water will be carried over with the organic solvent. They also provide a considerable saving in time and permit a number of extractions to be performed simultaneously. Two types of such extractors have been described, one for extraction with solvents lighter than water (benzene, ether, toluene, etc.) and the other with solvents heavier than water (chloroform, carbon tetrachloride, etc.).

When liberating the organic base from its salt, an inorganic base other than ammonium hydroxide should be used; since the volatile ammonia is easily carried over by the extracting solvent. For the liberation of organic acids from their salts, a nonvolatile mineral acid such as sulfuric acid is preferred to hydrochloric acid.

Palkin Extractors.—The Palkin extractors and their modifications have been used for liquid-liquid extractions where heat will not decompose the extracted substance.

Extraction with Centrifuge Tubes.—A very efficient method for the separation of liquid phases which tend to emulsify is the use of glass-stoppered centrifuge tubes of approximately 50-ml. capacity. This technique is best applied to solvents lighter than water. The authors (73) have first used this technique for aqueous extraction of the active ingredient from an ointment and subsequently it has been applied to nonaqueous methods.

Procedure.—The sample in aqueous suspension or solution is placed into a centrifuge tube, the free acid or base liberated, if necessary, and the proper organic solvent added. The stoppered tube is vigorously shaken and centrifuged to obtain complete separation of the layers. Most of the organic solvent layer is carefully withdrawn by means of a 10–20-cc. syringe, fitted with a cut-off blunt needle (preferably No. 13). This procedure is repeated a number of times until complete extraction is effected. The compound in the combined solvent extracts is titrated directly by nonaqueous methods.

Examples: Salts of Organic Acids:
- sodium benzoate
- sodium saccharin
- sodium diphenylhydantoin
- sodium pentobarbital capsules
- sodium phenobarbital tablets

Salts of Organic Bases:
- ephedrine sulfate capsules and ampuls
- amphetamine sulfate tablets
- quinine sulfate capsules
- codeine phosphate tablets and ampuls
- papaverine hydrochloride tablets and ampuls

Extraction of Phenol and Similar Compounds.—It is often necessary to remove and determine phenol in pharmaceutical preparations such as ampul or vial solutions, where it is added as a preservative. For the extraction of phenol or phenolic-type compounds in the presence of other organic acids, sodium bicarbonate or ammonium hydroxide is added to the sample in order to produce a salt, while phenol will not react with such weak bases. Phenol is then extracted with an organic solvent, a small quantity of ethylenediamine is added, and the ether evaporated to almost dryness. Additional ethylenediamine is added and the solution titrated with potassium methoxide using *o*-nitroaniline as indicator (28).

To separate phenols from organic basic compounds, the solution is acidified with dilute sulfuric acid, the phenol extracted with ether and determined in a similar manner.

TABLE V.—INTERFERENCES IN TITRATIONS

Titration of Acids
Weakly acidic substances
Water, which should be restricted to a minimum
Carbon dioxide, which can be held to a minimum by titrating in a closed system or under nitrogen
These precautions should be especially observed when titrating with weaker than 0.1 *N* solutions
Esters (ethyl acetate)
Some halogen containing compounds
Interferences from titrants, which form gelatinous precipitates
Titration of Bases
Weakly basic substances
Water and alcohol. Water above 1% causes poor end points and erroneously high results. These interferences in glacial acetic acid can be eliminated by boiling with acetic anhydride (except when primary or secondary amines are being titrated)
Most inorganic cations, except Hg and Cu
Titration of Reducible Substances
Alcohols, acids, amines, aldehydes, ketones, or any compound susceptible to reduction with lithium aluminum hydride
Water
Atmospheric oxygen. (These titrations must be carried out under nitrogen)
Brominations
Substances oxidizable with bromine
General Precaution.—Due to the considerably greater expansion coefficient of the titrant solvents, such as glacial acetic acid, *p*-dioxane, methanol-benzene, etc., in comparison to water, standardized solutions should not be subjected to significant temperature changes. Correction tables can be computed for volume changes due to fluctuations in temperature.

ACCURACY AND PRECISION

The accuracy and precision of a variety of nonaqueous titrations have been put to the test by a number of investigators. Seaman and Allen (74) compared titrations of sodium carbonate and potassium acid phthalate in glacial acetic acid, obtaining a precision of ±0.00008 for sodium carbonate and ±0.00009 standard deviation for potassium acid phthalate. An accuracy of ±0.2% was obtained in titrations of pure methylnicotinate. In a survey analysis of about 400 compounds, Markunas and Riddick (55) reported a precision of ±0.2%, using perchloric acid as titrant.

The authors (63, 64, 65, 72) also have shown that excellent accuracy can be obtained when comparing titrations of salts of organic bases and acids against standard procedures, with a precision of the same magnitude, as mentioned above. Fritz (21) likewise carried out similar comparisons of aqueous and nonaqueous titrations and reported a precision of ±0.2% for titrations in acetic acid and ±0.3% for titrations in basic solvents. The degree of precision depends largely upon the strength of the acid or base titrated. For the titration of relatively strongly acidic or basic compounds the precision reported by the above investigators can be easily obtained. When titrating very weakly acidic or basic compounds, such a precision should not be expected. Lintner, Schleif, and Higuchi (52) obtained an accuracy of 2 to 3% in electrometric titrations with lithium aluminum hydride.

TABLE VI.—NATIONAL FORMULARY IX COMPOUNDS

Acids and Their Salts	Bases and Their Salts
Acetarsone	Acriflavine HCl
Acetic Acid[a]	Aminoacetic Acid[a]
Amobarbital[a]	Aminopyrine Elixir[a]
Sodium Amobarbital[a]	Antipyrine[a]
Anthralin	Arecoline HBr[a]
Barbital Tinctures[a]	Brucine Sulfate[a]
Beta naphthol[a]	Butylaminobenzoate
Cinchophen	Citrated Caffeine
Dehydrocholic Acid	Chloramine T
Formic Acid[a]	Cinchonidine Sulfate
Guaiacol[a]	Cinchonine Sulfate
Halazone	Cocaine HCl[a]
Mandelic Acid	Codeine[a]
Parachlorophenol	Dibucaine HCl
Pentobarbital Elixir[a]	Glutamic Acid HCl
Phenyl Salicylate[a]	Histidine HCl[a]
Pyrogallol	Morphine HCl[a]
Resorcinol Monoacetate	Phenacaine
Salicylamide	Proflavine HCl
Stearic Acid[a]	Proflavine Sulfate
Sulfapyridine[a]	Quinine and Salts[a]
Thymol[a]	Racephedrine HCl
Trinitrophenol	Strychnine[a]
Undecylenic Acid	Theobromine[a]
	Inorganic Salts[a]

[a] Successfully titrated compounds.

TABLE VII.—UNITED STATES PHARMACOPEIA XIV COMPOUNDS

Acids and Their Salts	Bases and Their Salts
Acetylsalicylic Acid[a]	Aminopyrine[a]
Aminophylline[a]	Amphetamine[a]
Barbital[a]	Apomorphine HCl
Benzoic Acid[a]	Atropine and salts[a]
Bishydroxycoumarin	Aureomycin HCl[a]
Carbazone	Benzalkonium Chloride[a]
Citric Acid[a]	Butacaine Sulfate
Cresol	Caffeine
Dimercaprol	Carbachol
Diphenylhydantoin[a]	Chloroguanide HCl[a]
Folic Acid	Chloroquine Phosphate
Hexylresorcinol	Cocaine HCl[a]
Iodoalphionic Acid	Codeine Salts[a]
Iodochlorohydroxyquin	Colchicine
Iodophthalein Sodium	Dihydromorphinone HCl
Lactic Acid	Dihydrostreptomycin
Methyl Salicylate[a]	Diiodohydroxyquinoline
Methylparaben	Diphenylhydramine HCl
Nicotinic Acid[a]	Emetine HCl
Nikethamide	Ephedrine[a]
Pentobarbital Sodium[a]	Epinephrine
Phenobarbital[a]	Ergonovine Maleate
Phenobarbital Sodium[a]	Ergotamine and salts
Phenol[a]	Ethylaminobenzoate
Phthalylsulfothiazole	Ethylenediamine[a]
Propylparaben	Ethylmorphine HCl
Propylthiouracil	Eucatropine HCl
Saccharin[a]	Histamine phosphate[a]
Salicylic Acid[a]	Homatropine HBr
Sodium Benzoate[a]	Methacholine Chloride
Sodium Iodomethamate	Methamphetamine HCl
Succinylsulfathiazole[a]	Methenamine
Sulfadiazine[a]	Morphine Sulfate[a]
Sulfaguanidine	Naphazoline HCl
Sulfamerazine[a]	Neostigmine Bromide[a]
Sulfanilamide[a]	Nicotinamide[a]
Sulfathiazole[a]	Oxophenarsine HCl
Tartaric Acid[a]	Papaverine HCl[a]
Theophylline[a]	Para-aminobenzoic Acid[a]
Thiopental Sodium	Pentaquine Phosphate
Trichloroacetic Acid	Phenylephrine HCl[a]
Vanillin[a]	Physostigmine Salicylate
	Pilocarpine HCl
	Piperocaine HCl
	Procaine HCl
	Quinacrine HCl
	Quinidine Sulfate
	Quinine and Salts[a]
	Scopolamine
	Streptomycin base
	Tetracaine HCl
	Thiamine HCl[a]
	Tubocurarine Chloride
	Triethanolamine
	Tripelennamine HCl

[a] Successfully titrated compounds.

COMPOUNDS OF THE N. F. IX AND U. S. P. XIV WHICH MAY BE APPLICABLE TO NONAQUEOUS TITRATIONS

A subcommittee, headed by B. L. Clarke, has been recently established by the Contact Committee of the A. P. M. A. and A. D. M. A. in order to investigate the application of nonaqueous titrations to U. S. P. compounds. Listed in Tables VI and VII are compounds of the N. F. and U. S. P. believed to respond to nonaqueous titrations. Those so marked have been titrated successfully, while the others suggest possibilities for investigation.

TABLE VIII.—UNITED STATES PHARMACOPEIA XV ADMISSIONS

Acids	Bases
Amobarbital[a]	Chlorocyclizine HCl
Secobarbital	Chlorothen Citrate
	Chloroprophenpyridamine Maleate[a]
	Doxylamine Succinate[a]
	Methapyrilene HCl[a]
	Phenindamine[a]
	Pyrathiazine HCl[a]
	Pyrilamine Maleate[a]

[a] Successfully titrated compounds.

CONCLUSION

The field of nonaqueous titrations offers a variety of possibilities for the assay of pharmaceutical compounds, many of which cannot be determined readily by other methods. The authors have attempted to present the various techniques and procedures, reported up to the present time. Only in recent years have these methods found wide acceptance in research and control. Nonaqueous titrations present many opportunities for further theoretical and practical investigations. It appears that the time is not too distant when nonaqueous titrations will be used as frequently as titrations in water for the assay of pharmaceuticals.

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1954 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1954 Chilean Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee, including date of birth, and a list of his publications to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. Nominations must be received on or before January 1, 1954.

A nominee must be a resident of the United States or Canada. During the period covered by the nomination, he shall have been actively engaged in, have completed, or have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, he shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The award, consisting of $1,000 and a diploma setting forth the reasons for selection of the recipient, will be presented at the annual meeting of the ASSOCIATION. At this meeting, the recipient will deliver a paper or lecture upon the subject of his scientific work which will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. He will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee appointed by the chairman of the ASSOCIATION'S Council. It includes Justin L. Powers, chairman; Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Youngken, Lloyd M. Parks, and Paul Block, Jr.

Streptomycin Therapy in Nontuberculous Diseases*

By HERMINIE B. KITCHEN and SELMAN A. WAKSMAN†

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INTRODUCTION

IN THE FEW YEARS since its introduction to the medical world, streptomycin has become an important drug for the control of many infectious diseases in man and animals. Numerous reports of clinical successes with the drug are appearing daily in the medical literature.

Keeping abreast of this literature is a herculean task for the investigator, let alone the medical practitioner. He realizes full well that streptomycin has definitely established the possiblity of using chemotherapy in the treatment of various forms of tuberculosis caused by the acid-fast *Mycobacterium*. But he may be less familiar with the numerous findings on the effectiveness of streptomycin in the therapy of a wide variety of diseases caused by Gram-negative bacteria as well as by Gram-positive organisms that are resistant to penicillin and other chemotherapeutic agents. Furthermore, many internists have not yet become fully aware that with the relatively small dosages and the short-term treatments now known to be required to control nontuberculous infections, the untoward side reactions to streptomycin that were associated with early tuberculosis therapy are no longer a serious clinical problem.

* Received June 18, 1953, from the Institute of Microbiology, Rutgers University, New Brunswick, N. J.

† The authors are indebted to members of the medical staffs of the research laboratories, institutes, and divisions of several pharmaceutical companies for critical reading of the manuscript and for many helpful suggestions.

Bacterial nomenclature follows that of *Bergey's Manual*, 6th Edition, 1948. Some of the antibiotics mentioned are registered under trade marks, as follows: chloramphenicol, "Chloromycetin" by Parke, Davis & Company; chlortetracycline, "Aureomycin" by Lederle Laboratories of the American Cyanamid Company; oxytetracycline, "Terramycin" by Chas. Pfizer & Co.

Today, clinicians report that streptomycin is one of the safest and most effective antibiotics available for the treatment of many nontuberculous infections.

In a field as active as that of antibiotics, where clinical studies of both established and newly developed products are being continually initiated, the relative efficacy of the various antibiotics is subject to constant re-evaluation. Numerous pathogenic organisms once sensitive to penicillin and other antibiotics, for instance, have now developed resistance to these drugs. Combined therapy employing streptomycin and other drugs is one of the latest answers to this problem, though it may not be the ultimate solution. Other developments, as well, are being reported in the voluminous literature.

It would seem helpful to the medical profession, therefore, to make available in summary form the latest information on the extensive therapy of streptomycin, alone, and in combination with other drugs, in nontuberculous diseases. The first such summary was prepared in 1945 (THIS JOURNAL), when the total number of references was less than 60. Recently the Institute of Microbiology of Rutgers University published "The Literature on Streptomycin 1944–1952," comprising nearly 6,000 references dealing with this antibiotic. Since then, more than a thousand new references have appeared. Manifestly, not all reports of clinical investigations rate streptomycin high in comparison with other antibiotics and some record conflicting findings, but many show striking results. Digests of the more important of the recent reports of *effective* clinical uses of streptomycin in the therapy of human diseases other than tuberculosis are presented here in the hope that they will be of assistance to practitioners in the successful treatment of their patients.

CHEMICAL AND PHYSICAL PROPERTIES OF STREPTOMYCIN

Streptomycin is produced by a soil organism belonging to the actinomycetes and known as *Streptomyces griseus*. It has the capacity to inhibit the growth of and even to destroy numerous disease-producing bacteria. As a result of hydrogenation of streptomycin, a derivative was obtained designated as dihydrostreptomycin, which has antibiotic activity equivalent to that of streptomycin.[1]

Streptomycin, as the free base, as the chloride and sulfate salts, and as the calcium chloride complex, is extremely soluble in water. The base is insoluble in organic solvents, such as ether, chloroform, and acetone. The chloride is completely soluble in methanol, less soluble in ethanol, and virtually insoluble in butyl alcohol, acetic acid, and pyridine. The sulfate is only slightly soluble in methanol and virtually insoluble in the other organic solvents.

Both streptomycin and dihydrostreptomycin are extremely stable. The dry powder is stable[1] at room temperatures up to 30° C. (86° F.) for forty-eight months. Sterile solutions prepared from the dry powder form, in concentrations of 100 to 500 mg./ml., can be kept at room temperature for one week. Ready-to-inject solutions, usually containing 500 mg./ml., with added buffers, stabilizers, and preservatives, are stable at room temperature for at least twenty-four months. On aging, solutions of streptomycin and, to a lesser degree, dihydrostreptomycin tend to become darker yellow in color, but contrary to popular belief, this discoloration is not an indication of spoilage or loss of potency and has no effect whatever on the therapeutic value of the product. Because refrigeration delays this color change, which some persons consider psychologically objectionable, most pharmaceutical houses recommend that all solutions be stored in a refrigerator.

ANTIBACTERIAL ACTION

The outstanding feature of the action of streptomycin is its ability not only to inhibit the growth of but also to destroy various bacteria in the human and animal body as well as in the test tube. Its marked bactericidal activity against many pathogenic organisms places streptomycin in a highly favorable position among the antibiotics.

The antimicrobial action of streptomycin *in vitro* may be either bacteriostatic or bactericidal, depending on concentration of the drug, type of organism, nature of the medium, time of exposure, reaction of substrate (hydrogen-ion concentration), number of living cells used in the test, and temperature.

The effectiveness of streptomycin *in vitro* for inhibiting growth of microorganisms is usually expressed in terms of the smallest amount per milliliter of medium which completely prevents growth. Such concentrations of streptomycin are referred to as bacteriostatic and furnish a basis for comparing the activity against various microorganisms. Moreover, these concentrations give some indication of the effectiveness of the antibiotic for treating infectious diseases in the human and animal body.

Streptomycin is highly effective *in vitro* against a large number of pathogenic bacteria, Gram-negative, Gram-positive, acid-fast, and spirochetal organisms. Among the most susceptible Gram-negative bacteria are the *Pasteurella*, *Brucella*, *Hemophilus*, *Salmonella*, *Klebsiella*, and *Shigella* groups. The Gram-positive organisms most susceptible include *Streptococcus pyogenes* and the *viridans* group, *Micrococcus pyogenes* var. *albus* and *aureus* (*Staphylococcus albus* and *aureus*), *Diplococcus pneumoniae*, *Bacillus anthracis*, and *Corynebacterium diphtheriae*. The acid-fast organisms include strains of *Mycobacterium tuberculosis*.

Though susceptibility in the test tube is not necessarily the same as that in the human or animal body, there is a direct correlation between the two: organisms highly susceptible *in vitro* respond to the action of streptomycin *in vivo* more readily than do those moderately sensitive, and those very resistant *in vitro* do not respond at all *in vivo*.

[1] Throughout this publication, the term "streptomycin," except when otherwise specifically stated or implied, refers to both streptomycin and dihydrostreptomycin.

The activity of streptomycin in human and animal infections depends on the concentrations achieved at the locus of infection as well as on the susceptibility of the causal organism to the drug under given conditions. Such concentrations depend on the pharmaceutic form of streptomycin, route of administration, dosage, and condition of the patient. This last factor is most important in subacute or chronic infections in which tissue reactions may alter the supply of blood or other body fluids to the site of infection. The activity of streptomycin is decreased but little by body fluids, pus, or normal tissue juices. Its optimum activity takes place at a slightly alkaline reaction of about *p*H 8. Activity of the drug is inversely proportional to the concentration of viable organisms and is greatest against actively growing bacteria.

Certain bacteria exposed to any antibiotic for prolonged periods undergo marked changes in morphology and in resistance. The changes effected by exposure to streptomycin are more pronounced among Gram-negative than among Gram-positive organisms. Bacteria may develop resistance to streptomycin in two ways: gradually through a series of changes, or in a single step, the result of a single genetic change which produces a resistant variant. Recent evidence demonstrates that organisms do not develop resistance as a result of any action of the drug itself. Rather, various new types of organisms arise through spontaneous mutation, and through a process of selection, a resistant strain is set up.

One solution to the problem of the development of resistance appears to lie in the adoption of multiple antibiotic therapy, that is, the simultaneous administration of two or more antibiotics, such as streptomycin and penicillin in mixed infections of the urinary tract or streptomycin and one of the tetracyclines in brucellosis, or a combination of an antibiotic and a purely chemical compound, such as streptomycin and sulfadiazine in the treatment of human glanders, or streptomycin and PAS or INH in the treatment of tuberculosis.

Besides delaying or preventing bacterial resistance, as does a combination of streptomycin and penicillin in many infections due to Gram-negative intestinal bacilli, multiple antibiotic therapy often has other advantages. It may improve the prospects of cure in mixed bacterial infections, because of the individual action of several antibiotics against separate members of the mixed flora, as for example, streptomycin and bacitracin for intestinal antisepsis, or streptomycin and penicillin for peritonitis. It may prevent superinfection which sometimes occurs during single antibiotic therapy. Or the administration of smaller quantities of several compounds may prevent the side effects that often arise from large amounts of one antibiotic. An excellent example of this is the recently suggested use of a combination of streptomycin and dihydrostreptomycin, which reduces to a minimum the hazard of vestibular damage from streptomycin and of hearing loss from dihydrostreptomycin. Combinations of antibiotics may exert a synergistic effect, that is, an effect greater than that produced by administration of the same antibiotics individually in identical or larger quantities, as, for example, streptomycin and penicillin against certain streptococci, *Klebsiella* species, and pneumococci, or streptomycin, bacitracin, and polymyxin against *Endamoeba histolytica*.

Streptomycin offers potentialities for effective combinations with other antibiotics, especially penicillin. Combinations of streptomycin and penicillin have proved especially valuable in the treatment of infections caused by Gram-positive organisms that are resistant to penicillin. In *in vitro* studies with various combinations of antibiotics against bacteria that had developed resistance to penicillin and streptomycin, it has been reported that combinations of streptomycin and penicillin showed a synergistic effect against 37–90% of the Gram-positive organisms tested. Combined streptomycin and chlortetracycline exerted a synergistic effect against 40–91% of the Gram-negative bacteria, and streptomycin and chloramphenicol, against 44–100%. Of particular interest is the increasing synergistic effect as an organism develops resistance. In some instances, the combinations have an additive effect, as streptomycin, chlortetracycline, and chloramphenicol against *Pseudomonas aeruginosa* and *Proteus vulgaris*, or streptomycin and oxytetracycline or chlortetracycline against *Brucella*.

Some antibiotics in combination fail to react synergistically or even additively, but may have an antagonistic effect on one another. In other words, the effect of the combination may be less than the combined effects of the individual antibiotics. Recent work has shown that streptomycin is not among the drugs antagonistic to penicillin or to a variety of other antibiotics.

In the last few years, therapeutic application of mixtures of streptomycin and other antibiotics has been reflected in an increasing number of favorable clinical reports. Such combined therapy has proved of value in the treatment of endocarditis and bacteremia, surgical infections including peritonitis and extensive burns, gynecologic or obstetric infections, and other infections in many fields of medical practice.

PHARMACOLOGY

In the early days, a unit of streptomycin was designated as the amount that was just sufficient to inhibit growth of a standard strain of *Escherichia coli* in 1 ml. of culture medium. Later, when streptomycin was isolated in crystalline state, 1 unit was found to be comparable to 1 μg. of the pure base. The U. S. Food and Drug Administration, in consultation with the different manufacturers, established the potency of streptomycin on the basis of weight of active material. In referring to dosages of streptomycin salts, it has become customary to drop the expression "equivalent to milligrams of base" and speak of the dosage as "milligrams or grams of streptomycin."

Streptomycin and dihydrostreptomycin are supplied by pharmaceutical houses in syrups and in sterile, dry powder form for oral use or for preparation by the physician of solutions for injection. They are also available in ready-to-inject solutions.

Both streptomycin and dihydrostreptomycin may be administered by intramuscular injection. Some clinicians question the advisability of administering

dihydrostreptomycin intrathecally; in fact, the U. S. Food and Drug Administration recently suggested that manufacturers and labelers recommend streptomycin rather than dihydrostreptomycin for intrathecal therapy, except for patients allergic to streptomycin. For intrathecal and intravenous injection of streptomycin, only fresh solutions prepared from the dry powder form should be used. For intrathecal administration, the maximum amount that may safely be introduced into adults at any one time is 100 mg., smaller quantities being used at first. A solution containing 10–20 mg./ml. is commonly used. For intravenous administration, 1 or 2 gm. may be dissolved in 1,000 ml. of water for injection U. S. P. or sterile isotonic sodium chloride solution for parenteral use U. S. P. and administered at the rate of about 25 drops a minute. The concentration commonly recommended for aerosol use is 50–100 mg./ml.; for intrapleural or intraperitoneal use, 20 mg./ml.; for topical application in wet dressings or ointment form, 25–100 mg./ml.; and for oral solution, 100–200 mg./ml.

Absorption, Distribution, and Excretion

Absorption and excretion of streptomycin are not constant but vary in different patients and at different times in the same patient. After intramuscular or intravenous injection, streptomycin diffuses readily into most body tissues. After oral administration of doses recommended for therapeutic local effect in the gut (0.5 to 1 gm. in solution), streptomycin cannot be detected in the blood stream in significant amounts, and negligible concentrations appear in the urine. It is not destroyed in the gastrointestinal tract, and large portions of the orally administered drug can be recovered in the feces. Susceptible bacteria in the intestines are thus greatly reduced.

Gastrointestinal.—Oral administration of streptomycin is useful in preparing patients for surgical operations on the large intestine and in treating many gastrointestinal infections. It is one of the safest antibiotics for gastrointestinal therapy because it is not absorbed into the blood and therefore can have no toxic effect on the patient. Moreover, it does not destroy the entire bacterial flora of the intestine and therefore does not have the untoward effects often encountered in use of certain other antibiotics.

Urinary.—Satisfactory therapeutic concentrations appear in the blood and urine after intermittent intravenous or intramuscular administration. Intramuscular injection produces peak levels of streptomycin in the blood within one to three hours; the decrease is gradual over ten or twelve hours. If renal function is normal, about 60–80% is excreted in the urine within twenty-four hours, an important factor in providing adequate concentrations for treatment of urinary tract infections. Thus, the concentration and persistence of streptomycin in the blood are related to the dosage and renal function. It must be kept in mind, however, that the drug may accumulate in toxic amounts in the blood stream of patients with seriously impaired renal function.

Cerebrospinal.—Streptomycin does not diffuse readily into the cerebrospinal fluid of normal individuals, though therapeutic amounts may diffuse into the spinal fluid in presence of meningitis. Intrathecal as well as intramuscular injections are considered advisable, however, in bacterial meningitis. Intrathecal injections are made slowly at low concentrations in volumes slightly less than withdrawals after lumbar punctures. If intrathecal injection is followed within a few hours by marked increase in signs of meningeal or cerebral irritation, lumbar puncture should be done and spinal fluid withdrawn slowly if found to be under increased pressure.

Peritoneal.—Streptomycin appears to diffuse into the peritoneal cavity in substantial amounts in presence of early peritonitis. In advanced cases, both intramuscular and intraperitoneal injections may be required.

In Pregnancy.—Streptomycin diffuses through the placenta and thus to fetal circulation in concentrations about half those in the maternal blood. Evidence available suggests that streptomycin therapy during pregnancy is safe.

Biliary and Pancreatic.—Though partly excreted in the bile and pancreatic juice, streptomycin does not greatly alter the secretory activity of these organs.

Tracheobronchial.—When introduced into the tracheobronchial tree by nebulization or in powdered form, the drug can be administered to provide good local concentration and is not absorbed into the blood stream in significant amounts. When streptomycin and penicillin are combined for nebulization, the solution usually contains 50 mg. of streptomycin and 20,000 units of penicillin per ml. The use of penicillin, either alone or in combination with streptomycin for inhalation, has given rise to some sensitization.

Topical.—The clinician may occasionally find it desirable to apply streptomycin locally for the treatment of wounds infected by organisms susceptible to the action of the antibiotic. The high incidence of sensitization to streptomycin when applied topically has frequently led to use of dihydrostreptomycin instead.

Toxicity

With the small dosages and the short-term therapy required to control the nontuberculous infections, toxicity is rarely a clinical problem. Moreover, patients who are allergic to streptomycin can usually tolerate dihydrostreptomycin, and vice versa.

Streptomycin is less toxic to the auditory branch of the eighth cranial nerve, and dihydrostreptomycin is less toxic to the vestibular branch of this nerve, but neither deafness nor vestibular dysfunction is likely to develop in the treatment of nontuberculous diseases.

Allergic reactions such as drug fever, dermatitis, conjunctival irritation, and urticaria have been reported among patients, but they are infrequent, usually mild, and readily controlled by use of antihistamines. Nurses and physicians who administer streptomycin sometimes develop a contact dermatitis after handling the drug over a long period. Such reactions can be prevented by wearing rubber gloves.

CLINICAL INDICATIONS

Streptomycin is effective against diseases caused by most Gram-negative and many Gram-positive

bacteria. Recently, investigators have noted increasing incidence of micrococci (staphylococci) resistant to penicillin and the tetracyclines,—antibiotics commonly used in treating such infections. Moreover, there has been a marked increase in the proportion of strains of other bacterial pathogens resistant to the tetracyclines. In one study, for instance, half the strains of *Shigella* resisted these antibiotics. Bacteria that become resistant to chlortetracycline, oxytetracycline, or chloramphenicol are resistant to all three but not to streptomycin. Because of this increasing incidence of resistance to other antibiotics, as well as to sulfonamides, streptomycin therapy is indicated for many diseases caused by both Gram-positive and Gram-negative microorganisms for treatment of which other chemotherapeutic agents were formerly recommended.

The chief nontuberculous indications are as follows: bacteremia, meningitis, and urinary tract infections due to streptomycin-sensitive strains of *Aerobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa*, or *Salmonella* species; *Hemophilus influenzae* infections of the blood stream and heart, meninges, respiratory and urinary tracts; endocarditis due to penicillin-resistant organisms; pneumonia due to *Kl. pneumoniae;* empyema and other pulmonary infections due to streptomycin-sensitive organisms; chancroid; granuloma inguinale; gonorrhea unresponsive to penicillin; brucellosis; tularemia; plague; glanders; corneal ulcers caused by *Ps. aeruginosa*; wounds and cutaneous infections due to *Pr. vulgaris*; peritonitis and enteritis; prophylactically in gastrointestinal surgery.

In streptomycin therapy of many nontuberculous infections, an etiologic diagnosis is advisable, when practicable, to provide a guide to the dose of streptomycin and the duration of treatment. In most susceptible nontuberculous infections a daily dosage of 1–3 gm. administered intramuscularly in divided doses at intervals of four to twelve hours is effective. Treatment usually is continued for forty-eight to seventy-two hours after the temperature drops to normal and all signs of infection have disappeared. In the treatment of some infections, varying dosage schedules and different routes of administration give better results.

In mixed infections caused by Gram-positive and Gram-negative organisms a combination of streptomycin and other antibiotics or streptomycin and sulfonamides may be necessary. It is especially advisable, therefore, that these cases be studied etiologically if possible.

Once the diagnosis has been established, it is usually wise to determine the sensitivity of the causative organisms, though this may not be necessary in every case. In an acute infection, treatment should not be postponed until the study has been made.

THERAPEUTIC RESULTS

Bacteremia

Streptomycin therapy is successful in bacteremia when the causative organism is sensitive to the drug, when accessible foci of infection have been adequately drained, when the dosage of streptomycin and the intervals between doses are so spaced as to provide for bacteriostatic blood levels, and when treatment is continued long enough. It often clears the blood stream of infecting organisms and influences favorably the local lesions that provide the toxic foci. And when surgery is necessary, it provides protection during the operation.

A very common form of bacteremia is caused by *Proteus* organisms that invade the blood stream from the urinary and gastrointestinal tracts and from wounds. Most local infections are associated with serious lesions such as kidney or bladder tumors, diverticulitis, fecal fistulas, urethral stricture, nephrolithiasis, intestinal obstruction, and abscesses of skin and subcutaneous tissues. Some strains of *Proteus vulgaris* are particularly sensitive to streptomycin; in most cases success is attained with 2 gm. a day for five days. In some cases, streptomycin administered intravenously with either oral or intravenous oxytetracycline has proved effective. In patients who pursue a septic course after operation in the upper abdomen, infections are commonly caused by Gram-negative organisms. Blood specimens are taken for culture immediately and combined streptomycin and oxytetracycline or streptomycin and chlortetracycline are administered while the outcome of the test is awaited.

In bacteremia due to *Pseudomonas aeruginosa*, though polymyxin is commonly used, some clinicians prefer streptomycin.

In uncomplicated bacteremia caused by *Escherichia coli* or paracolon bacilli, many strains of which are sensitive to streptomycin, excellent results with this antibiotic have been reported. Because most strains of these organisms are also sensitive to chlortetracycline and oxytetracycline, combined therapy is most effective. This consists of intramuscular administration of streptomycin, 1 gm. twice daily, in combination with oral or intravenous use of either of the other antibiotics.

When *Aerobacter aerogenes* is the caustive organism of bacteremia, the primary focus is likely to be either the biliary or the urinary tract.

In *Klebsiella pneumoniae* bacteremia the commonest sites of primary infections are the lungs and the urinary tract. In such cases 1–3 gm. of streptomycin a day in divided doses for four to fourteen days is effective.

When *Hemophilus influenzae* is the offending organism, streptomycin is used.

Salmonella species invade the blood from the gastrointestinal tract or from local areas of infection in other parts of the body such as the lungs, urinary tract, or meninges. Streptomycin aids in clearing the blood stream and localizing the infection. Certain forms of *Salmonella*, notably those causing typhoid fever, are more effectively attacked by another antibiotic, such as chloramphenicol.

The portal of entry for Gram-positive organisms causing bacteremia is usually one of the following: operative and traumatic wounds; the uterus; urinary, gastrointestinal, and upper respiratory tracts; ear; mastoid; bone; or tooth extraction. Many of these organisms have become resistant to other antibiotics and can be eliminated by multiple antibiotic therapy including streptomycin.

The tendency to develop penicillin-resistance among the Gram-positive cocci, which are the most frequent causes of septicemia, occurs in the following descending order: *Streptococcus faecalis*, the *viridans* group of streptococci, and *Micrococcus pyogenes*.

For polymicrobic infections of the blood stream including microorganisms insensitive to penicillin, the preference of many clinicians is for combinations of streptomycin with penicillin or other antibiotics. In overwhelming acute bacteremic infections, immediate institution of streptomycin therapy is advisable before the etiological diagnosis has been established.

In *Brucella* and tularemic bacteremia streptomycin is the drug of choice. Against acute bacteremia due to *Brucella* species it is most effective when administered in combination with chlortetracycline or oxytetracycline.

Bacterial Endocarditis

By far the majority of cases of subacute bacterial endocarditis are due to infections by streptococci, especially *Str. salivarius* and *Str. mitis* of the *viridans* group and *Str. faecalis* of the enterococcic group. Other organisms known to cause bacterial endocarditis are *Hemophilus influenzae, H. parainfluenzae, Neisseria gonorrhea, Aerobacter aerogenes, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Brucella, Micrococcus* (*Staphylococcus*), and meningococcus species, and *Erysipelothrix*.

Many clinicians advise that in the therapy of subacute bacterial endocarditis, streptomycin alone or in combination with penicillin or other antibiotics be used for susceptible Gram-negative bacteria and also when the causative organisms are penicillin-resistant Gram-positive microbes including *Streptococcus faecalis* and other nonhemolytic streptococci.

The direct bactericidal effects of therapeutic agents, not merely their bacteriostatic action, are considered to be of great importance in the treatment of bacterial endocarditis. From the practical standpoint, however, several workers suggest that differentiation between the bacteriostatic and bactericidal action of the various antibiotics requires complicated laboratory tests and must usually be reserved for refractory cases. Use of antibiotics in combination also requires study. A practical procedure is to identify the organism responsible, usually a streptococcus, and ascertain the effect of various antibiotics *in vitro*. If the strain is susceptible to a given antibiotic, this fact is used as the basis of treatment.

Streptomycin and penicillin, because of their synergistic action, make a good combination for use in bacterial endocarditis caused by enterococci. Streptomycin reinforces the bactericidal action of penicillin, and the combination cures many cases resistant to penicillin alone. When the infection is caused by an enterococcus, therefore, streptomycin and penicillin therapy is instituted at once. The chief causes of failure are attributed by many clinicians to delay in treatment and inadequate therapy when bacteria are highly resistant. With delayed treatment, the recovery rate is found to be much lower.

In many infections of *Streptococcus faecalis*, combined streptomycin and penicillin therapy has made possible satisfactory clinical cure in two weeks. The therapy suggested is intramuscular injection of 0.5–1.0 gm. streptomycin morning and evening plus maximal doses of penicillin administered daily by intravenous drip. If combined streptomycin and penicillin are administered from the outset of treatment, one investigator favors a single injection of 2 ml. every twelve hours, each injection containing 0.5–0.6 gm. of streptomycin and adequate penicillin. This treatment can be continued for as long as four weeks without evidence of toxicity and with satisfactory clinical results, he says.

When the causative organism is *Str. salivarius* or *Str. mitis* and is sensitive to the streptomycin and penicillin combination, 60–70% of patients with subacute bacterial endocarditis recover after combined therapy for two to three weeks.

Infections caused by *Hemophilus influenzae* and *H. parainfluenzae* are treated with streptomycin intramuscularly and with oxytetracycline or chlortetracycline orally, according to some investigators. In the less common cases of bacterial endocarditis due to *Brucella* species, streptomycin combined with either of these two antibiotics also is the most effective treatment.

Recurrence of subacute bacterial endocarditis following discontinuance of antibiotic treatment is due in most cases to use of an antibiotic that is merely bacteriostatic. Moreover, discovery and suppression of bacterial foci not accessible to antibiotics are essential if relapses are to be prevented.

Subacute bacterial endocarditis is reported to be more prevalent in older groups than is generally suspected. The disease is often overlooked in elderly people because it shows symptoms suggestive of neoplastic disease, virus infections, pyelonephritis, and degenerative vascular disease.

Several investigators suggest that in tooth extraction, mechanical cleansing of teeth, and dental prophylaxis of patients with any history of rheumatic diathesis or with known valvular damage, streptomycin and penicillin prophylaxis should be instituted. As they point out, *viridans* streptococci are the predominant saprophytes of the human mouth and cause subacute bacterial endocarditis when the organisms gain access to the blood stream and then to damaged heart valves. In clinical cases, antibiotic prophylaxis resulted in an 82% reduction of bacteremia as compared with a 17.1% reduction in untreated patients. The combined therapy here comprised parenteral streptomycin 1 gm. and penicillin 400,000 units together with a troche of penicillin, bacitracin, and sulfadiazine. If the exodontist had difficulty in extraction, the troche alone was found to be valueless. When considered necessary after tooth extraction, a further parenteral dose of streptomycin and penicillin was recommended.

Meningitis

Streptomycin has proved to be the most valuable single therapeutic agent for the treatment of meningitis due to Gram-negative bacilli. It is effective also against penicillin-resistant Gram-positive coccal species that sometimes invade the meninges. Among the Gram-negative rod-shaped bacteria, the most important cause of meningitis is *Hemophilus influenzae*. *Escherichia coli* ranks second. Other rod-shaped Gram-negative bacteria that cause meningeal infections are *Pseudomonas aeruginosa, Klebsiella pneumoniae, Aerobacter aerogenes, Alcaligenes faecalis*, and *Salmonella, Proteus*, and *Brucella* species.

H. Influenzae Meningitis.—One of the most widely used and most effective remedies for *H. influenzae* meningitis is streptomycin. Any delay

in treatment greatly reduces the patient's chance of recovery. It is important, therefore, to identify the causative organism and determine its *in vitro* sensitivity immediately and meanwhile to begin intramuscular administration of streptomycin in divided doses of 1–2 gm. daily. In severe influenzal meningitis, use of sulfadiazine as an adjunct to antibiotic theory is recommended, and in some cases specific *H. influenzae* type B rabbit antiserum.

In very young infants mild to moderately severe influenzal meningitis has been cured by four to five days of treatment consisting of intrathecal administration of streptomycin together with injections of sulfadiazine and specific rabbit antiserum.

In a recent report on 90 cases of meningitis caused by *H. influenzae*, treatment with streptomycin was credited with reducing the mortality rate to 3.4%. In the majority of these cases, improvement took place within six days after treatment was started. Results were as good in patients treated intramuscularly alone as in those who received streptomycin intrathecally. Sulfadiazine was also given, but its value proved uncertain.

For *H. influenzae* meningitis, the usual daily dose employed for adult patients by most clinicians appears to be streptomycin 1–2 gm. in divided doses injected intramuscularly, with 25–50 mg. intrathecally. Intramuscular treatment is continued for at least eight to ten days. Some clinicians report that daily intrathecal administrations should continue for at least five to seven days; others report that a single intrathecal dose of 25 mg. is adequate.

Klebsiella Pneumoniae Meningitis.—This is not common, and streptomycin is effective in its treatment. A case has been reported of *Klebsiella* meningitis with liver abscess and recurrent bacteremia. Streptomycin cured the bacteremia but not the abscess.

Pseudomonas Aeruginosa Meningitis.—A number of reports dealing with *Pseudomonas aeruginosa* or pyocyaneous meningitis have recently appeared in the literature. This is a disease of high lethality which may occur as the primary form after spinal punctures or following traumatic lesions of the spinal fluid canal. It often follows chronic otitis media. Though systemic polymyxin with sulfonamides is widely used, streptomycin combined with sulfonamides, chloramphenicol, or chlortetracycline is reported to be effective against some strains of pyocyaneous bacteria. If meningitis due to *Pseudomonas aeruginosa* is suspected, tests to determine the causative organism are essential, and some clinicians recommend administration of streptomycin with sulfadiazine in adequate doses at once.

Streptomycin brought about recovery from primary meningitis due to *Pseudomonas aeruginosa* and *Neisseria flavescens* in a three-year-old boy.

Aerobacter Aerogenes Meningitis.—*A. aerogenes* is found principally in urinary tract infections, from which it may infect the meninges. A case of this type of meningitis with bacteremia following appendectomy with spinal anesthesia has been reported. Daily for five days 50 mg. of streptomycin was introduced into the spinal canal. The patient was also given 330 mg. of streptomycin intramuscularly every four hours for six and one-sixth days. The patient was discharged on the thirteenth hospital day free from all symptoms.

Salmonella Meningitis.—This is predominantly an affliction of infancy and childhood. Most cases have occurred in Central America, Cuba, and the southern United States, although isolated cases have been recorded in more temperate regions of the United States and in Canada. The most effective therapy seems to be combined streptomycin and sulfadiazine.

Micrococcus (Staphylococcus) Meningitis.—Meningitis caused by *Micrococcus pyogenes* var. *aureus* is one of the most infrequent forms of the disease but is also the deadliest. A combination of streptomycin, penicillin, and chlortetracycline given in full dosage at the beginning of therapy was reported to be most effective against a sulfadiazine-resistant strain.

Respiratory Infections

Hemophilus Influenzae Pneumonia.—In cases of pneumonia caused by *Hemophilus influenzae*, streptomycin is a drug of choice. The recommended dosage is 0.25–0.5 gm. intramuscularly every six hours. The action of streptomycin on type B *H. influenzae* is primarily bactericidal, and intramuscular administration over a maximum period of twenty-four hours will ordinarily suffice to obtain the greatest streptomycin effect, according to some clinicians. Use of sulfadiazine as an adjuvant is advisable to bring under control any resistant variant that may survive the bactericidal action of streptomycin.

Tracheobronchitis of Infants.—Several workers recommend that all young infants with tracheobronchitis due to *H. influenzae* be given streptomycin until throat cultures indicate withdrawal of the drug.

Klebsiella Pneumoniae Pneumonia.—Since the advent of streptomycin and its therapeutic use, many more patients have recovered from *Kl. pneumoniae* or Friedländer pneumonia than ever before. Many investigators consider streptomycin the most firmly established drug for treatment of this infection. Because the natural course of this type of pneumonia is toward necrosis and abscess formation, intensive treatment is started without delay. Dosages of 0.5 gm. streptomycin are given intramuscularly every four to six hours.

Clinicians who treated pneumonia caused by Friedländer's bacillus in infants reported that streptomycin was the most effective therapy. Throat cultures from seven patients showed pure growth of *Kl. pneumoniae*. In all patients the disease had an insidious onset with coughing and with vomiting or diarrhea or both. In another infant with *Kl. pneumoniae* infection of the lungs and multiple cavities, combined streptomycin and chlortetracycline therapy sterilized the sputum and closed all cavities.

Pseudomonas Aeruginosa Infections.—Infections of the respiratory tract caused by *Pseudomonas aeruginosa* are relatively unimportant. Streptomycin with sulfonamides is effective.

Mixed Pulmonary Infections.—These are most likely to be met in chronic suppurative bronchitis, bronchiectasis, aspiration pneumonia, postoperative pneumonia, bronchial pulmonary fistulas, empyema, and lung abscesses. In such cases *in vitro* sensitivity tests on cultured bacteria are usually essential.

Depending on the results of these tests, aerosol forms of streptomycin in dosages of 0.1 gm. four times a day together with penicillin are helpful supplements to other forms of antibiotic therapy according to some reports. As several clinicians recently noted, Gram-negative organisms made resistant to other antibiotics retained their sensitivity to streptomycin, suggesting the possible importance of administration of streptomycin after use of the tetracyclines or chloramphenicol. Empyema caused by streptomycin-sensitive organisms may be treated by supplemental intrapleural installation of 0.1 gm. streptomycin and use of streptokinase and streptodornase in addition to systemic antibiotic therapy. Early and adequate use of proper antibiotics may abort or minimize suppurative pulmonary lesions and prevent development of lung abscesses in predisposing clinical situations such as coma and convulsions.

Infective Bronchial Asthma.—Dihydrostreptomycin is frequently used as an aerosol in cases where penicillin-resistant organisms are found in the sputum. The total daily dose ranges from 0.5 to 1.5 gm. In one report of 52 patients treated with penicillin aerosol, 10 failed to respond. When streptomycin was given by aerosol, the Gram-negative flora in the sputum disappeared, dyspnea diminished, and the vital capacity increased on the second or third day.

Bronchiectasis and Chronic Bronchitis.—Occasional development of Gram-negative organisms during prolonged treatment of chronic bronchopulmonary infections can be controlled by streptomycin or by short oral or intravenous courses of other antibiotics. Some clinicians prefer inhalational therapy and report that addition of streptomycin to penicillin may result in quicker response. Both aerosol and dust treatments have been used. Of twenty courses of combined streptomycin (0.2 to 0.4 gm. daily) and penicillin dust given by inhalation, the response was satisfactory in thirteen. Most clinicians point out that no antibiotic aerosol should be given unless there is evidence of purulent infection of bacterial origin and the possibility of side effects in allergic patients is taken into account.

More successful outcome of surgical treatment of bronchiectasis has been reported when patients were prepared by bronchoscopic drainage and nebulized instillation of solutions of streptomycin (125 mg. per milliliter, 1 ml. four times a day) and penicillin for four days preceding surgery. Other clinicians recommend intramuscular as well as aerosol penicillin preoperatively and intramuscular streptomycin (250 mg. every three hours) with penicillin for five to seven days after operation or until the temperature, pulse, and respiration become normal.

Hemophilus Pertussis.—A number of investigators have found streptomycin to be particularly effective in early stages of whooping cough in infants and small children, especially if the disease is uncomplicated. Others have found streptomycin effective in whooping cough with tuberculosis at various stages of the disease. In whooping cough with pneumonia, streptomycin with sulfonamides and penicillin shortened the duration of the disease and reduced mortality. For infants suffering from whooping cough with pulmonary complications, dosages of 0.3–0.75 mg. were given daily for two to four weeks. Daily treatment with 200 mg. streptomycin in an aerosol in three divided doses until symptoms were relieved was also reported effective for children. To very sick children, streptomycin was given parenterally as well, and when complications were present, penicillin and vaccine were also administered.

In 15 patients three months to seven years old, treatment of whooping cough with streptomycin was reported effective. These patients were given streptomycin intramuscularly 1 gm. daily in eight doses for a total of five days. Three other patients with bronchial pneumonia received streptomycin for eight days. The cough decreased in the first three days, and all patients were cured between the fifth and tenth day after initiation of therapy.

Pneumonic Tularemia and Plague.—Streptomycin has proved to be the most valuable antibiotic in the treatment of tularemic and plague infections.

Coccal Infections.—In respiratory diseases due to Gram-positive organisms that are sensitive to streptomycin, such as *Diplococcus pneumoniae*, combinations of streptomycin with sulfadiazine have been found effective. In streptococcal pneumonia, combinations of streptomycin with penicillin also have been used effectively.

Atypical Pneumonia.—Striking improvement has been reported in some cases of viral or atypical pneumonia treated with streptomycin. Some investigators report successful treatment of pneumonia of unknown etiology with combined streptomycin and penicillin. One patient was given 0.5 gm. streptomycin every six hours plus penicillin for two days. The second patient received penicillin for nine days; on the seventh day, streptomycin therapy, 0.5 gm. every six hours, was instituted. After administration of 5 gm. of streptomycin, the temperature began to fall and there was gradual progressive improvement.

Urinary Tract Infections

Urinary tract infections are caused by a variety of organisms. Of the Gram-negative bacteria, the most serious offenders are *Escherichia coli*, *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Alcaligenes faecalis*, and paracolon organisms. Others implicated are pleuropneumoniae groups of *Hemophilus influenzae*. Gram-positive bacteria infecting the urinary tract are chiefly micrococci (staphylococci) and streptococci of the nonhemolytic enterococcus and alpha-hemolytic groups. Though a single organism may cause urinary tract infection, mixed infections of two or more organisms are far more common.

Streptomycin is a highly valuable antibiotic for the treatment of most urinary tract infections. It has produced spectacular results in many cases. Patients with cystitis have responded well to it and it has been used with success in acute epididymitis, gonococcal urethritis, prostatitis, and infections of the bladder associated with paraplegic conditions.

Parenterally administered, streptomycin is rapidly absorbed and excreted in the urine in relatively high concentrations. In urinary tract infections susceptible to treatment with streptomycin, the urine is commonly sterilized within two to three days. Exceptions are due generally to nonsensitivity of the organism or to obstruction in the urinary tract.

Administration of 1 gm. streptomycin intramuscularly daily in divided doses every three to six hours commonly ensures a concentration of 100 μg./ml. of urine. This treatment for five to seven days usually proves effective, especially when the urine is alkalinized with 2 gm. of sodium bicarbonate or 2 gm. sodium citrate every four hours. When *Pseudomonas aeruginosa* or *Streptococcus faecalis* is present, the minimum dosage of streptomycin advisable appears to be 1.5 to 2 gm. daily. If *Proteus* species are present, alkalinization of the urine may be unnecessary. The patient's fluid intake, according to some clinicians, should be restricted to 2,500 ml. daily. Furthermore, if sodium bicarbonate or sodium citrate is used to alkalinize the urine, fluid retention of cardiac patients should be watched carefully following the sodium intake.

Therapeutic failures are more common with pathologic-anatomic abnormalities of the genitourinary tract which prevent the free flow of urine and allow accumulation of residual urine. Foreign bodies in the tract, such as calculi and indwelling catheters, offer convenient portals of entry for reinfection and predispose to development of resistant bacteria. Undrained abscesses and other inaccessible foci and wounds with granulating surfaces or fistulas communicating with the urinary tract prevent sterilization of the urine. Use of streptomycin alone in presence of localized accumulations of exudate may lead to resistance even in the short-time therapy required. Clinicians have reported, however, that streptomycin, when used in rational doses, is no more likely to induce resistance than is any other antibiotic, and some have expressed the opinion, further, that streptomycin is the most valuable antibiotic in most urinary tract infections.

Use of streptomycin with catheter drainage is not effective. Such drainage should be limited to uncontrolled acute upper urinary tract disease or to bacteremia associated therewith. Lower urinary tract infection with persistent pyuria is one of the troublesome and frequent complications of transurethral prostatic resection. In such cases streptomycin is not very effective.

The most recent clinical reports emphasize the value of combined antibiotic therapy, the careful selection of proper medication as indicated by culture methods, and prior elimination of any factor that mitigates against success, such as calculus or stasis, and finally, employment of generous dosages of medication.

Mixed infections are more persistent than single-organism infections. The clinical usefulness of streptomycin with penicillin or other antibiotics directed toward treatment of mixed infections caused by Gram-negative and Gram-positive organisms in urinary infections is emphasized by many investigators. Combined antibiotic therapy is especially effective where *in vitro* tests indicate that no single agent will cover all the pathogens. Combinations have also been reported effective when two antibiotics are observed to act synergistically *in vitro* against a single bacterial species and when the combination allows appreciably smaller quantities of one or both antibiotics to be used. The following antibiotic combinations have been recommended for urinary tract infections: those caused by *E. coli* and *A. aerogenes*, streptomycin, chlortetracycline, and chloramphenicol; *Proteus vulgaris* and *Pseudomonas aeruginosa*, streptomycin and chloramphenicol.

A recent report discussed use of *in vitro* sensitivity studies in calculating a rational dosage of antibiotics in 17 cases of urinary tract infections treated with satisfactory clinical and bacteriological results. No resistance developed to any of the antibiotics studied (streptomycin, penicillin, chloramphenicol, chlortetracycline, oxytetracycline), and the number of resistant replacement strains was less than with the usual standard doses of antibiotics. These results suggested that if no more than the lowest effective dose is used, the infecting organism is unlikely to become resistant. According to this report, a case of pyelonephritis, the infecting organism being *E. coli*, was cured with a dosage of 1 mg. of streptomycin every six hours. Renal calculus with pleural effusion caused by *E. coli* was cured with 7 mg. every six hours. A case of acute pyelonephritis with enterococcal and micrococcal infecting organisms was cured and the urinary tract rid of bacteria by combined streptomycin and penicillin therapy. Streptomycin was also found effective against *E. coli* in acute cystitis, in pyelonephritis with diverticulitis, and in pyelonephritis of pregnancy.

Another recent report pointed out that therapy with penicillin in huge amounts failed when several strains of micrococci resistant to penicillin secreted penicillinase; streptomycin treatment was curative.

Venereal Infections

Three venereal infections, chancroid, gonorrhea, and granuloma inguinale, can be treated successfully with streptomycin.

Chancroid.—In the treatment of chancroid, which is caused by *Hemophilus ducreyi*, streptomycin is an antibiotic of choice. Good results have been obtained with 0.5 gm. administered intramuscularly every four hours for eight doses. Some clinicians report that in severe cases streptomycin combined with chlortetracycline or with sulfadiazine gives better results than does streptomycin alone. Others have found that even in refractory cases, especially when patients are sensitive to the sulfonamides or to chlortetracycline, streptomycin alone has been satisfactory; one report showed 100% cure of hospitalized cases.

Granuloma Inguinale.—This infection is caused by *Donovania granulomatis*, or Donovan bodies, and is generally considered to be a venereal disease acquired through coitus. One of the minor scourges extant throughout the southern United States, it is reported from all parts of the world, especially tropical and subtropical areas. Streptomycin is the most effective drug for treatment. Many cases have been cured with as little as 0.5–1 gm. a day administered intramuscularly in divided doses every six hours for five days. Donovan bodies disappeared from the lesions and healing took place seven to thirty days after cessation of therapy.

Gonorrhea.—Infections due to strains of *Neisseria gonorrhea* unresponsive to penicillin or in patients allergic to penicillin have been treated successfully with streptomycin. Some clinicians prefer streptomycin to penicillin for the treatment of gonorrhea because penicillin is likely to mask a syphilitic infection, which often accompanies gonorrhea. The full advantage of penicillin is thus reserved for the more serious infection. Gonorrhea and its complications usually respond to a single dose of 0.3–0.5

gm. of streptomycin in aqueous solution injected into the gluteal muscle, repeated once if necessary. Acute symptoms disappear rapidly after treatment; the patient may be considered cured when routine tests remain negative for six months. Gonorrheal vulvovaginitis in girls three to seven years old was reported cured by injection of 2–5 gm. of streptomycin in doses of 0.2 gm. every three hours.

Gastrointestinal Infections

Antibiotic therapy in gastroenterology has two main objectives, namely, prevention of infection after abdominal surgery and control of infectious diseases involving the digestive tract. Rational use of chemotherapeutic agents in the treatment of intestinal infections and in preoperative and postoperative management of patients undergoing surgery of the bowel has reduced morbidity and mortality in a spectacular manner, according to clinicians.

Peritonitis.—Many physicians have had marked success in the treatment of peritonitis with antibiotics and sulfonamides. This dreaded complication of so many diseases now usually responds to intelligent and vigorous use of these drugs when combined with proper surgery and general supportive care. Streptomycin has decreased mortality from peritonitis caused by inflammatory and perforating lesions of the gastrointestinal tract, especially infections of a single Gram-negative organism or a mixed Gram-negative flora. When possible, cultures of infecting organisms are tested for sensitivity to various drugs. But even in absence of this laboratory aid, physicians can institute a therapeutic regimen with confidence. In many cases, streptomycin has been found effective in dosages of 1 gm. twice daily until the temperature is normal and then 0.5 gm. daily for one week. Combinations of streptomycin, penicillin, and sulfonamides usually produce better results, however, in delaying or preventing bacterial resistance and, because of their synergistic effect, in suppressing the fecal flora.

Ulcerative Colitis.—Some investigators believe that in ulcerative colitis, the chief value of antibiotics is in the control of secondary infections which may culminate in abscess formation and peritonitis. They report that combinations of streptomycin and penicillin with sulfadiazine may be effective. Clinical improvement has been reported occasionally in ulcerative colitis treated with bacitracin alone or together with streptomycin and glucurolactone. Bacitracin together with streptomycin and glucurolactone eliminated Gram-positive bacteria and decreased the number of *Escherichia coli*. Several investigators have reported striking results with streptomycin, given orally for ulcerative colitis; the drug reduced the number of bacteria in the feces and altered for about a week the type of organisms present. Simultaneous use of two or more compounds is considered to be more effective, however, than administration of the same compounds individually in identical or larger amounts.

Bacillary Dysentery.—The causative agents of bacillary dysentery are usually *Shigella* species. Streptomycin by both parenteral and oral routes has been highly successful in treatment of the infection. Because the antibiotic is absorbed in negligible amounts after oral administration, this route is reported especially advantageous for local effect within the intestine. Orally, 0.5 gm. is usually given five times a day; intramuscularly, 1 gm. twice a day, the dosage being reduced later to 0.5 gm. twice daily. In fulminating cases, immediate use of streptomycin and sulfadiazine together has been helpful. In cases resistant to sulfonamides or in patients sensitive to the sulfa drugs, streptomycin is effective alone; combinations with glucurolactone have been used with some success.

Amebiasis.—Some investigators have found streptomycin with bacitracin and polymyxin effective against amebiasis caused by *Endameba histolytica* infestation. Others report successful synergism of streptomycin with penicillin and the sulfonamides.

Intestinal Diseases of Infancy.—Experiences recently reported with 70 infants with gastroenteritis at the Pediatric Clinic in Naples led to the conclusion that streptomycin, chloramphenicol, and chlortetracycline were helpful in severe cases of dysentery. Sulfonamide therapy was considered advisable in milder cases.

Another report concerned streptomycin treatment of 109 infants, including dystrophic and premature babies, with diarrhea. Of the dystrophic patients, 45 were suffering from diarrhea of the dysentery type. Streptomycin was given in a dose of 150 mg. per kg. body weight, 100 mg. administered orally and 50 intramuscularly. In some cases the total amount was given in divided doses. Good results were obtained. In premature babies with diarrhea, streptomycin therapy effected prompt recovery.

In 36 cases of acute enterocolitis in infants, *Salmonella* organisms were found in 64% of the stool cultures and *Proteus* in 28%, associated in one case with *Shigella* and in two cases with *Salmonella*. In five cases *Shigella paradysenteriae* was identified. The blood culture from one child yielded *Salmonella kauffmannii;* in this case streptomycin was administered parenterally 50 mg. every four hours. In all other cases the administration was oral, 25 mg. every three hours for the first twenty-four hours and 50 mg. later for infants less than six months old. Older patients received 50 mg. every three hours from the beginning. The mortality rate was less than 3%, and best results were obtained on *Salmonella* and *Proteus* cultures.

Outbreaks of infectious diarrhea of the newborn attributed to atypical coliform organisms and other bacteria including *Pseudomonas aeruginosa, Proteus vulgaris,* and *Streptococcus faecalis* have yielded to oral and parenteral administration of streptomycin.

Prophylaxis in Gastrointestinal Surgery

Streptomycin individually and in combination with other chemotherapeutic agents has proved of great value in suppressing the fecal flora of the intestinal tract prior to surgery and thus in reducing the incidence of postoperative infection. Streptomycin has decreased the mortality from peritonitis caused by inflammatory and perforating lesions of the gastrointestinal tract, especially infections of a single Gram-negative organism or a mixed bacterial flora.

To be effective, the antibiotic must come in contact with the pathogens and remain in contact with them for an appreciable time. Intestinal obstruction interferes with the results. Constipation pre-

vents the drug's coming in contact with the pathogens, and diarrhea prevents its remaining in contact long enough. Loculated pus, as in fistulas and abscesses, is difficult to sterilize. In these cases sulfonamides are of distinct value but are reported to be inferior to streptomycin, chlortetracycline, and oxytetracycline.

Favorable results with streptomycin as an adjunct to surgical treatment have been described in occasional instances of acute cholecystitis, cholangitis, pyelophlebitis, and liver abscesses when Gram-negative intestinal bacteria were the infecting organisms.

In preoperative and postoperative management of patients undergoing surgical procedures on the bowel, streptomycin is used extensively. Doses of 0.5 gm. given four times daily by mouth or parenterally decrease the total bacterial content of the feces chiefly by inhibiting *E. coli;* yeasts and the anaerobes persists. The suppressive effect occurs rapidly, but the duration is relatively short. Bacterial counts usually increase again after four to six days, since streptomycin does not "sterilize" the intestine. In this respect, the drug avoids certain of the untoward effects of some other antibiotics.

Daily administration of 2 gm. of streptomycin and 8 gm. of glucurolactone in four divided doses for five days prior to resection of the bowel has been suggested. The last doses are given two hours prior to surgery. Susceptible coliform bacteria have been suppressed in all cases reported. Maximum suppression was obtained in sixty hours to the fifth day of treatment. Some investigators have reported oral administration of these drugs to result in fecal sterilization for as long as six weeks.

Many clinicians prefer combined streptomycin and penicillin therapy for prophylaxis prior and subsequent to surgery of the bowel. As they point out, in the preparation and protection of surgical areas, streptomycin combined with penicillin has proved clinically useful when directed toward the treatment of mixed infections caused by Gram-positive and Gram-negative microorganisms.

Wounds and Cutaneous Infections

Combined streptomycin and penicillin therapy as an adjunct to surgery has been found beneficial in chronic osteomyelitis unresponsive to penicillin alone. In most of the cases reported, the wounds contained a mixed flora; *Micrococcus pyogenes* var. *aureus* was the most common Gram-positive organism observed, and *Proteus vulgaris* the most common Gram-negative. Surgery comprised radical sequestrectomy with sauceration of the involved area. Streptomycin and penicillin were administered in varied dosages according to severity of infection and sensitivity of the organisms. Antibiotic therapy was started the day before surgery and continued for ten days. After sequestrectomy, the wounds were left undisturbed for fourteen days and the dressings were changed weekly under aseptic technique. Streptomycin and penicillin were applied locally at each dressing. Of 25 patients thus treated, 21 were healed.

Gram-negative bacteria usually dominate the flora of infected wounds and definitely interfere with their healing. Topical administration of a solution containing 200 μg. of dihydrostreptomycin per milliliter together with penicillin usually has been more effective in preventing wound infections than in hastening resolution of an established infection. In wounds infected with a mixed bacterial flora, streptomycin has been used topically with penicillin to kill Gram-negative bacteria that elaborate penicillinase, which destroys the penicillin.

In skin infections, immediate benefit from use of streptomycin has been observed when the infecting organism is sensitive to streptomycin. In general, such cases include pyodermas due to Gram-positive organisms, stasis ulcers, and dermatophytosis secondarily infected with Gram-positive and sensitive Gram-negative organisms. The method of administration depends on the type and extent of the disease. Parenteral administration is usually used for cutaneous infections that are extensive or essentially systemic in character. For infections of limited extent and more strictly cutaneous in nature, streptomycin is applied topically in a grease cream or gel base in concentrations of about 5 mg. per gm. at least four times a day. Prior cleaning of the skin with soap and water is suggested and all crust scales and other detritis are removed. Careful watch is kept for cutaneous sensitization. Should the patient prove to be sensitive to streptomycin, dihydrostreptomycin may be substituted, or vice versa.

Infections of the Eye, Ear, and Nose

In infections of the cornea, lesions are highly destructive. For this reason, early treatment is essential. Fortunately, the characteristic clinical appearance of such lesions facilitates early diagnosis. Streptomycin is effective when used subconjunctivally in infections of the cornea due to Gram-negative bacteria. It is also valuable locally in acute conjunctivitis due to such organisms.

In corneal ulcer caused by *Pseudomonas aeruginosa* and in otitis externa and media where *Ps. aeruginosa* is the secondary invader, combined streptomycin and sulfonamide therapy has been recommended.

In the treatment of 20 patients suffering from keratoconjunctivitis, excellent results were reported with streptomycin, 20–30 mg./kg. body weight daily for thirty days.

A case of acute eczematous dermatitis of the ear canal, caused by *Ps. aeruginosa*, was reported cured with an aqueous solution of streptomycin containing 2.5 mg./ml. After ninety-six hours the ear was free from secretion and the lesions were in process of healing. A follow-up study seven months later showed the patient definitely cured.

Other reports indicate that streptomycin offers tremendous help in the control of selected ear infections. It allows safer surgical intervention, and provides a more uneventful postoperative course in those diseases of the ear that are not readily controlled by sulfonamides and other antibiotics. Combined streptomycin, penicillin, and sulfonamides control the disease process long before an etiologic diagnosis can be made, because the combination covers both Gram-positive and Gram-negative organisms. Suppurative infections of the middle ear, antrum, or mastoid are surgically drained, and therapy is continued postoperatively where indicated. Topical use of streptomycin has been effective in many reported cases; combined topical and intramuscular therapy has been recommended for deep-seated infections.

In chronic maxillary sinusitis, surgical drainage through an antral window in the middle meatus combined with preoperative and postoperative administration of streptomycin and penicillin was reported to be the treatment of choice in one hospital. The combined antibiotic therapy was given for one or two days prior to drainage and for duration of hospitalization, usually four to six days.

Rhinoscleroma, caused by *Klebsiella rhinoscleromatis*, was successfully treated with a total of 28 gm. streptomycin, administered 0.25 gm. every four hours. Clinical improvement was noted in five days after start of therapy, and cultures became negative promptly. No lesions reappeared during a three-year period of observation. The disease, which is a granulomatous infection involving the nose and nasopharynx, occurs in Egypt, Eastern Europe, Central and South America, Asia, and Hawaii.

Miscellaneous Infections

Actinomycosis.—The common cause of actinomycosis is *Actinomyces bovis*. Clinicians have reported arrest of the disease by streptomycin therapy in several cases of systemic actinomycosis not benefited by penicillin. Streptomycin was administered intramuscularly 2 gm. daily in four divided doses during the first month, 1 gm. daily during the second month, and 0.5–1 gm. daily during the last month.

Anthrax.—This infection is caused by *Bacillus anthracis*, and has been effectively treated by streptomycin. Some workers suggest use of this antibiotic in cases that do not respond to penicillin.

Asiatic Cholera.—Prompt reduction of the number of infecting organisms (*Vibrio comma*) in stools and slight shortening of attacks have been reported with streptomycin therapy. Strains of cholera vibrios have been found to vary in sensitivity to antibiotics according to the place of origin of the organism. Those from Egypt and Bombay were said to be sensitive to a variety of antibiotics; those from Bengal and Assam, resistant. Vibrios from patients in one Chinese village were reported sensitive to amounts of streptomycin varying from 1–500 μg./ml.

Brucellosis.—This is also known as undulant fever, and is a systemic infection caused by *Brucella abortus*, *Br. suis*, or *Br. melitensis;* it is of worldwide distribution. Though primarily a disease of cattle, hogs, and goats, it is acquired by man as a result of drinking infected milk or handling infected animals. In all suspected cases, culture of the blood, stools, or enlarged lymph glands and agglutination, opsonocytophagic, or skin tests are recommended.

It appears to be generally agreed among clinicians that simultaneous use of streptomycin and chlortetracycline or of streptomycin and oxytetracycline yields the best results with all types of brucellosis. The rate of symptomatic and bacteriological relapse has been lower and the undesirable toxic reactions encountered in earlier methods have been eliminated. Pointing out that the defense mechanism of the host plays an important part in recovery from brucellosis, clinical investigators recently suggested that more successful eradication of *Brucella* organisms from tissues of infected human patients could be expected with a longer course of treatment than the twelve to fourteen days formerly recommended. For acute *Brucella* bacteremia, the recommended treatment consists of intramuscular administration of 2 gm. of streptomycin daily given every morning and evening and oral administration of chlortetracycline or oxytetracycline. For chronic brucellosis complicated by localizing culturally proved lesions, the daily dosage of streptomycin is halved and the combined antibiotic treatment is administered for twenty-eight days. In treatment of subacute bacterial endocarditis due to *Brucella* species, the most effective combination also has been found to be streptomycin and either of the tetracyclines.

In a recent report of 23 cases of acute or subacute brucellosis, 22 of which showed cultures of *Br. melitensis*, 1 gm. streptomycin intramuscularly daily together with oral oxytetracycline for a three-week period was effective. One patient ten years old was treated with half doses of both antibiotics. All patients were observed for four to seventeen months after completion of therapy. All were kept in hospitals for thirty days, during which 12 or more cultures of urine were made. Only 14% relapses were noted with this combined therapy for twenty-one days, in contrast to an 80% relapse rate noted in a previous study. Clinical response was good, and acute symptoms were adequately controlled under the prolonged therapy period. The authors concluded that the total period of combined therapy should be at least three weeks for *Br. melitensis*.

Carrion's Disease.—This is restricted to the western portion of South America and is found especially in Ecuador, Peru, and Colombia. It is a specific infection caused by a rickettsial-like organism, *Bartonella bacilliformis*, transmitted by certain sandflies. Two forms of the disease occur: the severe, often fatal Oroya fever accompanied by anemia, and the cutaneous Verruga peruana characterized by nodular cutaneous eruptions. Both streptomycin and penicillin have been found of value in treatment of the anemic stage; streptomycin is reported to be more efficacious in the eruptive stage than is penicillin.

Gangrene.—The synergistic effect of streptomycin, penicillin, and sulfonamides is of value when one drug alone fails to arrest the gangrenous process. Morbidity resulting from anaerobic streptococcic gangrene may be decreased by early recognition of the condition and by administration of streptomycin before the gangrenous process becomes extensive; incisions and skin grafting will then be unnecessary. These conclusions were drawn in a report on streptococcic gangrene of the breast in two women. Culture of material from the lesions showed hemolytic streptococci. Treatment with penicillin and sulfonamides failed to prevent spread of the infection. When streptomycin was added to the dosage, the infectious process was localized within a few hours, and incisions were not necessary.

Glanders.—Human glanders, caused by *Malleomyces mallei*, occurs rarely. It has been successfully treated with combined streptomycin and sulfadiazine. In one patient, 2 gm. streptomycin was injected daily for ten days and sulfadiazine was administered orally. The temperature then became normal, the sulfadiazine was discontinued, and 1 gm. of streptomycin was injected daily for seven more days, after which the patient was discharged as cured. In several other cases, a total of 50 gm. of

TABLE I.—BACTERIAL SENSITIVITY TO STREPTOMYCIN *in Vitro*[a]

Gram-Negative Bacteria		
Organism	Sensitivity Range (Streptomycin, μg./Ml. Medium)	
Aerobacter aerogenes	0.3	256.0
Brucella abortus	0.5	3.8
Br. melitensis	0.5	128.0
Br. suis	0.5	2.5
Erysipelothrix muriseptica	2.5	...
Escherichia coli	0.015	256.0
E. communior	1.0	8.0
Hemophilus ducreyi (Ducrey's bacillus)	1.0	15.0
H. influenzae	0.5	50.0
H. parainfluenzae	2.5	...
H. pertussis	1.2	50.0
Klebsiella ozaenae	0.5	1.5
Kl. pneumoniae (Friedländer's bacillus)	0.55	128.0
Kl. rhinoscleromatis	0.9	...
Neisseria gonorrheae (gonococcus)	1.0	40.0
N. meningitidis (meningococcus)	1.0	40.0
Pasteurella lepiseptica	0.5	2.5
Past. pestis	0.5	6.0
Past. tularensis	0.15	0.4
Proteus vulgaris	1.0	128.0
Pseudomonas aeruginosa (*B. pyocyaneus*)	0.1	500.0
Ps. fluorescens	4	12.5
Salmonella enteritidis (Gärtner's bacillus)	0.004	32.0
S. paratyphi	2.0	8.0
S. pullorum	1.0	16.0
S. schottmülleri	0.004	32.0
S. typhosa	0.004	20.0
S. typhimurium	0.004	32.0
Shigella paradysenteriae	0.25	10.3
Vibrio comma (*V. cholerae*)	5.0	500.0

Gram-Positive Bacteria		
Organism	Sensitivity Range (Streptomycin, μg./Ml. Medium)	
Bacillus anthracis	0.25	10.0
B. mycoides	0.13	8.0
B. subtilis	0.056	128.0
Corynebacterium diphtheriae	0.375	200.0
C. pseudotuberculosis	1.0	...
C. pyogenes	0.25	10.0
C. renalis	0.5	2.5
C. xerose	1.0	250.0
Diplococcus pneumoniae (pneumococcus)	8	60
Mycobacterium tuberculosis	0.15	300
Micrococcus pyogenes var. *albus* (*Staphylococcus albus*)	0.03	256.0
Micrococcus pyogenes var. *aureus* (*Staph. aureus*)	0.03	256.0
Streptococcus fecalis	1.0	100
Str. pyogenes	2.0	16.0
Str. lactis	16	...
Str. salivarius	5	25
Miscellaneous		
Actinomyces bovis	3.75	...

[a] Organisms vary greatly in their susceptibility to streptomycin, not only from one genus or species to another, but from strain to strain. This wide range in sensitivity is shown in the table.

streptomycin was administered to each patient; of these, three recovered and one relapsed after seven months and later died.

Leprosy.—Streptomycin is reported to be effective in the therapy of leprosy, caused by *Mycobacterium leprii*. In doses of 1 gm. intramuscularly, it was found to be a useful adjunct to sulfone therapy. According to some investigators, it enhanced the regression of specific leprous lesions; its effect on mucous membrane lesions was especially rapid, as improvement was noted in such lesions after a week or two of treatment. Much of this improvement was believed to be due to action of the drug on secondary invaders. Streptomycin is said to be capable of healing leprous ulcers of the mucous membrane and also of causing distinct regressive changes in leprous skin lesions after about six weeks of treatment. It has been reported that streptomycin has antileprotic properties also. Many cases of acute iridocyclitis have responded favorably, but far-advanced eye lesions are apt to progress unfavorably, as with sulfones.

Streptomycin has been found useful in treating cases in which improvement in skin and mucous membrane lesions appeared to have been retarded on sulfone therapy. When streptomycin was added to the sulfone treatment, skin lesions showed renewed clearing and mucous membrane lesions healed, relieving the patient of nasal obstruction from scabbing and crusting of the mucous membrane. Streptomycin is as effective as the sulfones in reducing organisms in the skin, studies for as long as two years have indicated. Treatment with the antibiotic cannot be continued as long as that with sulfones.

Plague.—This is caused by *Pasteurella pestis*. It is widely scattered throughout the world, and is endemic in several states of the United States. Streptomycin is reported to be the most valuable antibiotic in the treatment of this disease. It has dramatically reduced mortality rates in both bubonic and septicemic plague and has proved to be the most effective drug in pneumonic plague. One of the latest reports suggests intramuscular administration of streptomycin 4 gm. daily in divided doses every three to four hours. The dosage may be reduced on the third or fourth day of recovery. Sulfadiazine may be combined with the streptomycin therapy for seven to ten days. In severe septicemia and particularly in pneumonic plague, the initial dose of streptomycin is sometimes effectively supplemented by oral administration of chloramphenicol, chlortetracycline, or oxytetracycline and anti-plague immune rabbit globulin.

Radiation Injuries.—That exposure to both ionizing radiation and thermal trauma causes a high mortality has been found by analysis of the causes of death among the inhabitants of Hiroshima following atomic bombing. Though for obvious reasons

no clinical reports are available, recent tests with swine exposed to both roentgen radiation and flash burns showed that streptomycin administered parenterally beginning twenty-four hours after trauma and continued for twenty-one days reduced mortality from 90% to 20%. The streptomycin-treated animals showed no hemorrhage from mouth or bowel and only two showed petechiae of the skin. The investigators believe that streptomycin exerts a beneficial effect by reducing the extent of secondary infection and hemorrhage of the gastrointestinal tract. They concluded that early and adequate treatment with streptomycin will contribute to reduction of mortality in swine following a moderate amount of total body radiation combined with flash burn and that healing of second degree burns is accelerated. Streptomycin did not stimulate recovery of blood-forming tissue.

Rat-bite Fever.—This is caused by two different organisms, *Spirillum minus* and *Streptobacillus moniliformis* (*Actinomyces muris*). The disease caused by *S. minus* is also known as "sodoku"; that caused by the *Streptobacillus* is often called "Haverhill fever." Reports indicate that streptomycin has brought about recovery from both forms. A case in French Guinea, found to be due to *Spirillum minus* was cured by injection of 1 gm. streptomycin daily for ten days. In two days adenitis and eczema disappeared. From the second day of treatment the temperature was normal. In rat-bite fever caused by *Streptobacillus moniliformis*, which is characterized by joint involvement, satisfactory results have been reported with a dose of 1–2 gm. daily until the patient has been asymptomatic for two to three days.

Tularemia.—Infections caused by *Pasteurella tularensis* are widespread in the United States and have also been reported in Japan, Norway, Austria, Czechoslovakia, Sweden, and Turkey. From 95–98% of all tularemic infections are acquired from skinning or cleaning wild rabbits. Recent studies in the Ozarks have shown that the curative value of streptomycin is greater than that of tetracyclines. All forms of tularemia, including the glandular, ulceroglandular, oculoglandular, typhoidal, and pneumonic, are controlled promptly and effectively by streptomycin. Though disease of any duration will respond well to streptomycin therapy, the treatment is best instituted as early as possible. There is no evidence that early treatment interferes in any way with development of the usual solid immunity after recovery. In the pulmonary and typhoidal forms where the temperature remains elevated without localizing signs, 0.5–1 gm. streptomycin daily intramuscularly in divided doses until infection is under control has been recommended. For the typhoidal clinical type, typhoidal peritonitis, lymphadenitis, and subpectoral abscess, a dosage of 0.5 gm. a day for two days followed by 0.25 gm. a day for four days, all injections at intervals of eight hours, or intramuscular injection of 0.5 gm. a day at three- to four-hour intervals for six days has been suggested.

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Suppository Bases. I. An Evaluation of the Rates of Release of Theophylline*,†

By CHARLES F. PETERSON‡ and ARTHUR J. GUIDA§

A method is described for the evaluation of suppositories by allowing the drug to be released from the base and dissolved into normal saline solution at body temperature. The rate of release of the theophylline (as aminophylline) was compared for twenty-five suppository base formulas. Modifications of the water-soluble bases with certain disintegrating agents gave the most rapid release of the theophylline. Storage for one year showed a marked reduction in the rate of release of theophylline from the cocoa butter suppositories. The comparison of the theophylline release from the suppository when compounded as aminophylline, glycophylline, or theophylline sodium acetate, showed that each compound requires a specific base formula to allow the most rapid release of the drug. This test showed that slight changes in the physical-chemical properties of the drug-base relationship may influence the rate of release of the drug.

THEOPHYLLINE double salts are administered by various routes of administration. In emergency treatment, intravenous injection is preferred, although the compounds may be given orally, intramuscularly, by rectal instillation, or by rectal suppository. In measuring protection against intravenous histamine, Segal, *et al.* (1), demonstrated that the peak was reached within two hours after a rectal retention enema of aminophylline. The protection period lasted for two and one-half hours by this route which was twenty minutes longer than by intravenous administration. The tablets administered orally produced protection for less than one hour.

Waxler and Schack (2) found that the rectal suppository required four hours to reach a peak concentration which was only two-thirds of that after intramuscular injection of aminophylline. Truitt, *et al.* (3), confirmed these reports and showed that aminophylline by rectal retention enema gave the highest blood level of theophylline except for intravenous injection. The rectal suppository gave lower levels than the other routes of administration which included tablets by mouth in the same and in a lower dose. The difference in the rate of absorption of the theophylline from the retention enema and the suppository may be explained by the fact that the drug is dissolved in 15 to 20 cc. of water for the enema, and the drug in the suppository must first be dissolved in the limited fluid present in the rectum. The total absorptive area exposed to the solution of the drug is also much greater in the case of the enema. The suppository has the advantage of convenience and the possibility of maintaining a prolonged level of the drug.

EXPERIMENTAL

The suppositories were made by the fusion process to contain 0.5 Gm. aminophylline. Each suppository was wrapped and stapled snugly within a piece of filter paper[1] for the test. The filter paper cover was to minimize the difficulty of having oil drops from the fatty bases from interfering with the withdrawal of the samples. It also lengthened the time for the drug to dissolve and pass into the solution, acting as a barrier to maintain a uniform area of contact between the water and the base.

The wrapped suppository was placed in a 50-cc. beaker and covered with 10 cc. of normal saline solution. The beaker was covered with a watch glass and placed in a constant temperature bath maintained at 37.5° ± 0.2°. At intervals a sample was withdrawn, the volume being maintained by the addition of an equal amount of normal saline solution. The theophylline content was determined by the method reported by Plummer (4). The principle of this assay is the precipitation of copper theophyllinate from an alcoholic solution separation, and iodometric titration of the copper.

TABLE I.—FATTY TYPE SUPPOSITORY BASES

Base No.	Formula
1	Cocoa butter
2	Cocoa butter plus 8% yellow wax
10	Propylene glycol monostearate[a]
23	Propylene glycol monostearate[b]
16	Stearyl alcohol and 40% oleyl alcohol[c]

[a] Kessler Chemical Corporation, Philadelphia, Pa.
[b] Emcol PS-50, Emulsol Corporation, Chicago, Ill.
[c] U. S. pat. 2,586,329, February 19, 1952, to E. K. Harvill.

RESULTS

Table I lists the formulas for the fatty type bases. Figure 1 shows the curves representing the per cent of the total theophylline released with time from these bases. The time scale is divided

[1] E & D No. 613, Eaton-Dikeman Company, Mount Holly Springs, Pa.,

* Received January 5, 1953, from the School of Pharmacy, University of Kansas, Lawrence, Kans.
Presented before the Pharmacy Subsection (Np) of the American Association for the Advancement of Science, St. Louis meeting, December 29, 1952.
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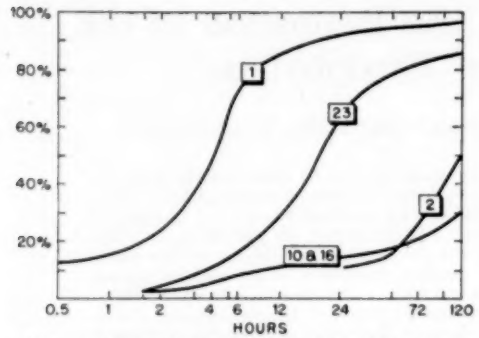

Fig. 1.—Theophylline release from fatty bases.

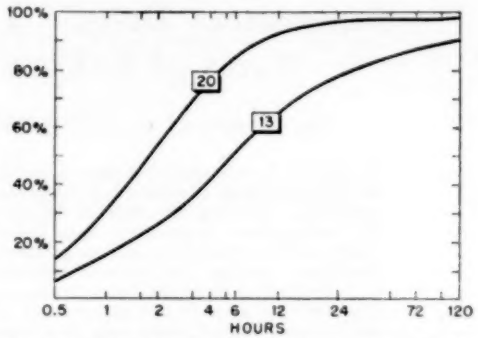

Fig. 2.—Theophylline release from water-soluble bases.

logarithmically to emphasize the initial portion of the curves.

Table II lists the formulas for the water-soluble bases in an approximate ranking by the theophylline release rate. The time for 50% release of theophylline was calculated graphically and is reported to the nearest one-quarter hour. Figure 2 shows the curves for the theophylline release from base No. 20 and No. 13, the highest and lowest, respectively, of the water-soluble bases.

After storage of the suppositories for approximately one year, during which the temperature varied from 15° to 37°, nine of the suppositories were tested for their theophylline release. Only those suppositories considered satisfactory after storage were given this test. Figure 3 shows the release rates for the cocoa butter suppositories after storage compared to those freshly prepared (these were tested within three days after preparation). Of the water-soluble bases, there was a decrease in the rate of release of the plain Carbowax 4000 base (No. 3) after storage for one year. The results are given in Table III. The other bases tested in this manner were 4, 5, 6, 8, 9, and 11 and they did not demonstrate a different rate of release (the 50% release time did not differ by more than one-quarter hour).

TABLE III.—EFFECT OF STORAGE ON THE THEOPHYLLINE RELEASE RATE, AMINOPHYLLINE SUPPOSITORY, CARBOWAX 4000 BASE

Time in Hours	Per Cent Released	
	Fresh[a]	Stored[b]
0.5	9	8
1.0	20	24
1.5	30	29
2.0	39	34
3.0	53	44
4.0	59	50
5.0	67	58
24.0	86	77
48	91	84
72	91	86
96	92	89
120	93	89

[a] Tested three days after preparation.
[b] Tested after one year storage.

TABLE II.—WATER-SOLUBLE SUPPOSITORY BASES

Hours for 50% Release:	Base No.	Carbowax[a] 1540	1500	4000	6000	Water	Other Ingredients
1 3/4	20	...	10	70	...	10	10% sodium sulfate ex.
2	19	...	10	80	...	5	5% sodium sulfate ex.
2	22	33	...	...	47	10	10% sodium sulfate ex.
2 1/2	8[b]	...	10	85	...	5	.....
2 1/2	4[b]	...	10	90	...	...	.....
2 1/2	6[b]	...	15	85	...	...	.....
2 1/2	21	33	...	...	47	...	20% P E G 400[e]
2 3/4	3[b]	...	...	100	...	...	.....
3	9[b]	...	10	80	...	10	.....
3	15	...	...	88	...	10	2% Veegum[c]
3	17	...	10	80	...	9	1% C M C high viscosity[d]
3 1/4	18	...	10	80	...	9	1% C M C medium viscosity[d]
3 1/4	5[b]	...	5	90	...	5	.....
3 1/4	25	...	10	80	...	...	10% propylene glycol
3 1/2	11[b]	...	10	89.5	...	...	0.5% Methocel 4000[e]
4	12	...	10	89	...	...	1% Methocel 4000[e]
4	14	...	10	...	80	10	.....
4	24	...	10	85	...	...	5% propylene glycol
4	7	...	10	...	75	15	.....
5 1/2	13	...	10	85	...	...	5% cetyl alcohol

[a] Carbon and Carbide Chemicals, New York, N. Y.
[b] Also tested after one year storage.
[c] R. T. Vanderbilt Company, New York, N. Y.
[d] Hercules Powder Company, Wilmington, Del.
[e] Dow Chemical Company, Midland, Mich.

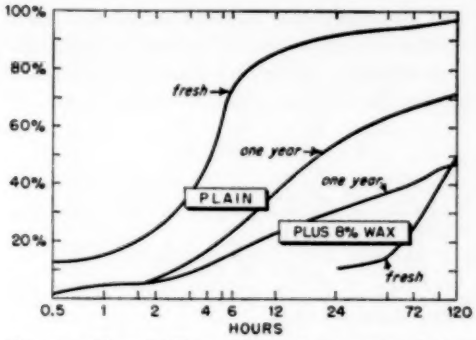

Fig. 3.—Theophylline release from cocoa butter after storage.

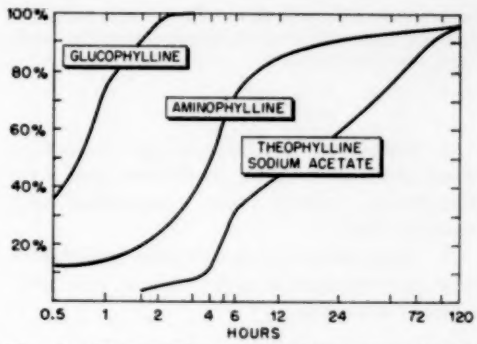

Fig. 4.—Theophylline release from cocoa butter comparison of the different salts of theophylline.

Five of the bases were selected for comparison of the theophylline release rate when the suppositories are compounded with the different salts of theophylline. Theophylline sodium acetate, and glucophylline[2] were compounded into the suppositories in the same dose (0.5 Gm.) as the aminophylline so that the same amount of powder was incorporated into the base. Table IV shows that the release rate from the same base containing different compounds is not consistent, that is, each salt of theophylline favors a different base for the maximum release.

TABLE IV.—THEOPHYLLINE RELEASE FROM BASES USING DIFFERENT SALTS OF THEOPHYLLINE

	Time in Hours for 50% Release		
Base No.	Gluco-phylline	Amino-phylline	Theophylline Sodium Acetate
1	3/4	4	16
3	1 3/4	2 3/4	1 3/4
4	1	2 1/2	4 1/2
15	2	3	4 3/4
19	1 1/4	1 3/4	5

DISCUSSION

The twenty-five bases reported are those which were successfully made by the fusion process. Among the chief difficulties was that Carbowax 4000 has a tendency to form a gelatinous mass which could not be poured into the mold. Neither theophylline alone nor Phyllicin (Bilhuber-Knoll) gave this gel formation, but the other forms of theophylline did show this difficulty. Inclusion of 10% Carbowax 1500 or polyethylene glycol 400 reduced the tendency, but it was noted upon prolonged heating. Soaps, potassium, sodium, and triethanolamine stearates were found to be incompatable with the aminophylline by the formation of a mass that could not be melted. The self-emulsifying propylene glycol monostearate cannot be used for this reason. The various modifications were introduced to influence the release of theophylline by causing the base to disintegrate more quickly in water. The sodium sulfate with water appeared to be a better substance for this purpose than water alone, as this mixture allows a slight increase in the release rate over the plain Carbowax mixture. The other additions tended to decrease the release rate, possibly by increasing the consistency of the base. These results confirm part of the work by Hassler (5) who had chosen formulas for rectal suppositories of sodium phenobarbital on the basis of their dissolution time in water. The release rate for his base (formula No. 21) was improved by the incorporation of the sodium sulfate-water mixture for the polyethylene glycol 400.

The changes in the release brought about by storage of the cocoa butter are interesting. The partial explanation offered is that the ethylene diamine present in the aminophylline reacted with the free fatty acids. These soaps have been noted above as being incompatible, causing a marked increase in the melting point of the suppository mass. The stored suppository made with a base containing 8% wax showed an increase in theophylline release over that of a freshly prepared one. In this case it is possible that other changes in the physical characteristics occurred which allowed the theophylline to be more readily released. Perhaps the wax prevented the soap formation and aging allowed the theophylline to migrate to the surface.

The most interesting observation in this study is that theophylline salts are released at different rates from the same base. This observation must be made with the realization that the theophylline double salts contain differing amounts of theophylline and are soluble at differing amounts. Each suppository was compounded with 0.5 Gm. of the drug (the usual dose) but aminophylline contains about 78%, glucophylline about 50%, and theophyline sodium acetate about 60% theophylline. The per cent theophylline release was based on the total available in the suppository. One hundred per cent theophylline release by the glucophylline suppository may be compared to 80% for theophylline sodium acetate or 60% for aminophylline.

The plain Carbowax 4000 base showed the least variation with the different salts, while cocoa butter showed the greatest variation. Glucophylline was released by each of the bases most rapidly followed by aminophylline and theophylline sodium acetate.

This method has been used to screen various formulas of suppositories to note some of the factors which influence the release of the drug from the base so that it may be absorbed by the body. Further studies are necessary to establish the relationship between the results of this test and what actually

[2] Supplied by the Abbott Laboratories, North Chicago, Ill.

occurs in rectal absorption. The *in vitro* technique is useful as a screening technique where the conditions must be controlled.

SUMMARY

1. Carbowax base suppositories containing water and sodium sulfate showed the most rapid theophylline release when compounded with aminophylline.

2. Suppositories made with plain cocoa butter base demonstrated a slower release rate after storage for one year.

3. Glucophylline, aminophylline, and theophylline sodium acetate are released in that order from different suppository bases, but the ratio between the three are different for each base.

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Solubility of Phenobarbital in Propylene Glycol-Alcohol-Water Systems*

By CHARLES F. PETERSON and RAYMOND E. HOPPONEN

The solubility of phenobarbital is reported in combinations of the solvents propylene glycol, water, and alcohol. The solubility reaches a maximum at a 50 per cent mixture of propylene glycol and alcohol (anhydrous). Propylene glycol, 100 per cent, dissolves more phenobarbital than 100 per cent alcohol. Upon addition of water to the solvents, the solubility falls off more quickly with propylene glycol than with alcohol. The results show that it is possible to prepare an elixir of the same concentration of phenobarbital as the official elixir without the use of alcohol or glycerin. Several elixirs were made and the principal difficulty noted was that the characteristically poor taste of phenobarbital was accentuated by the acrid taste of propylene glycol.

THE SCARCITY of glycerin during World War II and the threat of scarcity in the event of increased military demands has prompted the trial of substitute solvents in many pharmaceuticals. In the case of elixir of phenobarbital, propylene glycol and combinations of propylene glycol and other poly-alcohols have been suggested as substitutes, and formulas using these substitutes have been proposed (1, 2). However, no systematic study of the solubility of phenobarbital in propylene glycol combinations has been reported in the literature. It was felt that as a basis for developing a substitute formula a study of the solubility of phenobarbital in mixtures of propylene glycol, alcohol, and water should be made.

As a method of analysis, the potentiometric titration with sodium hydroxide as proposed by Waters, Berg and Lachman (3) and modified by Mattocks and Voshall (4) was adopted. Krause and Cross (5) have reported an accuracy of about 0.5 per cent for the method. When applied to known samples of phenobarbital in mixtures of propylene glycol, alcohol and water, we also found the accuracy to be of the order of 0.5 per cent.

* Received January 5, 1953, from the School of Pharmacy, University of Kansas, Lawrence, Kans.
Presented before the Pharmacy Subsection (Np) of the American Association for the Advancement of Science, St. Louis meeting, December 29, 1952.

EXPERIMENTAL

Solubility Determination.—A series of 66 solvent combinations was made from propylene glycol, absolute alcohol, and water. Concentrations of propylene glycol and alcohol ranged from 0 to 100% by volume in increments of 10%. Where the combined volumes of propylene glyco and alcohol were less than 100%, the balance was water. Saturation of the solution with phenobarbital was insured by adding an excess of phenobarbital to the solvent contained in a 60-cc. screw-capped bottle which was sealed with paraffin and rotated for sixty hours in a water bath at a temperature of 25° ± 0.5°. Duplicate samples of 15 cc. of the saturated solution were analyzed by adding an excess of 0.1 *N* sodium hydroxide and titrating to a *p*H of 10.5 with 0.1 *N* sulfuric acid. A Beckman Model H2 *p*H Meter equipped with a calomel and a Type "E" glass electrode was used to indicate the end point. A blank was run on each solvent combination and a suitable correction made. Results are reported in Table I and illustrated graphically by Fig. 1

TABLE I.—SOLUBILITY OF PHENOBARBITAL IN PROPYLENE GLYCOL-ALCOHOL-WATER SYSTEMS

Per Cent Alcohol	Per Cent Propylene Glycol										
	0	10	20	30	40	50	60	70	80	90	100
0	0.14	0.19	0.22	0.40	0.59	1.09	1.82	3.38	5.85	9.28	14.95
10	0.17	0.28	0.45	0.79	1.29	2.41	4.07	6.86	10.19	15.90	
20	0.28	0.58	0.89	1.76	2.89	4.91	7.69	11.62	16.85		
30	0.56	1.17	2.03	3.59	5.56	8.55	11.93	17.48			
40	1.37	2.39	4.15	6.26	9.26	13.49	17.65				
50	2.88	4.72	7.31	10.43	13.57	17.75					
60	5.19	8.13	10.70	13.46	17.18						
70	8.25	11.52	13.78	16.64							
80	11.23	13.45	15.38								
90	13.10	13.86									
100	11.79										

NOTE.—Where the sum of propylene glycol and alcohol does not equal 100, the balance is water.

Fig. 2 illustrates in more detail that portion of the solubility data which includes the concentration of phenobarbital generally used in elixirs.

Formulation.—On the basis of the results obtained, the formulas listed in Table II were prepared. The syrup content was increased from the 15% of the official formula to 20% to aid in masking the bitter taste of phenobarbital since propylene glycol does not add any sweetness as does the glycerin of the official formula.

The formulas were subjected to storage stability tests for twelve weeks under the following conditions: at room temperature in screw-capped bottles, in the refrigerator at 5°, in the refrigerator and at room temperature on alternate days, in half-filled 240-cc. bottles loosely stoppered with cotton. A sample of the official elixir of phenobarbital prepared at the same time was subjected to the same conditions. None of the samples stored at room temperature in screw-capped bottles showed any change. Formula 1 stored in the cotton-stoppered bottle showed crystallization at the end of the twelve week storage period. Crystals appeared in the sample stored in the refrigerator as follows: No. 1 after seven days, No. 2 after twenty-six days, No. 3 after seventy-four days, No. 5 after eleven days, No. 6 after five days, No. 7 after sixty-four days. When returned to room temperature, the crystals in all but Nos. 1 and 5 went back into solution within three days. Of the samples alternated between the refrigerator and room temperature, only No. 1 (after six days) and No. 5 (after seventy-one days) showed any crystallization.

The substitute formulas were not as palatable as the official elixir because the acrid taste of propylene glycol contributes little masking effect. It was felt that perhaps a more tart flavor than that of the official elixir might blend more readily

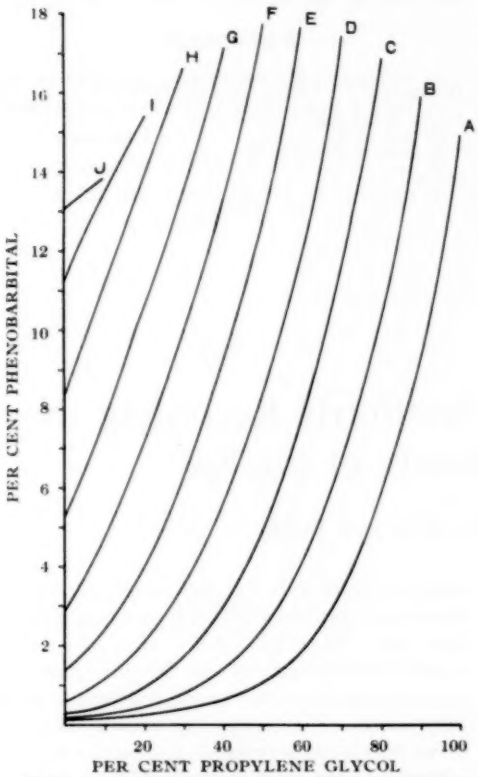

Fig. 1.—Solubility of phenobarbital in propylene glycol-alcohol-water mixtures. Per cent of alcohol, A—0%, B—10%, C—20%, D—30%, E—40%, F—50%, G—60%, H—70%, I—80%, J—90%.

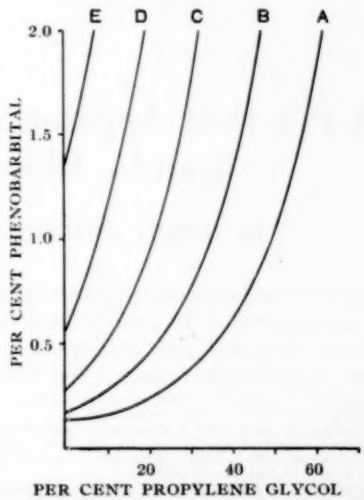

Fig. 2.—Solubility of phenobarbital in propylene glycol-alcohol-water mixtures. Per cent of alcohol, A—0%, B—10%, C—20%, D—30%, E—40%.

TABLE II.—PHENOBARBITAL ELIXIR FORMULAS USING PROPYLENE GLYCOL

Formula	1	2	3	4	5	6	7	8
Phenobarbital	4	4	4	4	4	4	4	4
Alcohol	100	100	100	100	...	...	...	...
Propylene Glycol	150	200	250	300	250	300	350	400
Syrup	200	200	200	200	200	200	200	200
Sweet Orange Peel Tincture	30	30	30	30	30	30	30	30
Amaranth Solution	10	10	10	10	10	10	10	10
Distilled Water to make	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000

with the acrid taste of the glycol and the bitterness of the phenobarbital. Three test elixirs containing 35% of propylene glycol, 20% of syrup, 0.4% of phenobarbital, 0.1% of a flavoring oil and 0.5% of citric acid to impart a tartness were prepared. Terpeneless oils of orange, lemon, and lime were used. In the case of the lime oil, it was necessary to use 1% of a 10% spirit in order to get satisfactory solution of the oil. None of the elixirs was colored to prevent possible rejection of the lime flavor because of a departure from the traditional green color usually associated with it. An official elixir of phenobarbital with color omitted was used for comparison. These elixirs were checked for acceptability by placing five drops on the back of the hand which aided in spreading the elixir on the tongue when transferred to the mouth. A group of 31 senior pharmacy students served as test subjects. Only two samples were checked at a time, the subject simply designating which of the two was least unpleasant. Results are reported in Table III.

TABLE III.—FLAVOR PREFERENCE

	PER CENT
Official elixir over lemon	88
Official elixir over lime	79
Official elixir over orange	77
Lime over lemon	68
Lemon over orange	55
Lime over orange	52

SUMMARY

1. The solubility of phenobarbital in propylene glycol-alcohol-water systems ranging in concentrations from 0 to 100% of propylene glycol and of alcohol has been determined.

2. Substitute formulas for elixir of phenobarbital based on these data have been prepared and subjected to storage stability tests.

3. Taste tests showed that the propylene glycol containing preparations were less acceptable than the official elixir.

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A Practical Apparatus for Indirectly Recording the Systolic Blood Pressure of the Rat*

By HENRY DOUGLAS JOHNSON† and ELBERT VOSS‡

A TAIL PLETHYSMOGRAPH apparatus for indirect measurement of the systolic blood pressure of the rat was constructed for use in an experimental hypertension project. The better features of several of the tail plethysmograph designs described in the literature (1–5) were incorporated into our apparatus. With two exceptions, namely, the pressure cuff and pressure gauge, all parts were made from materials available in most laboratories. No special techniques such as metal working were required, this being a decided advantage over most of the published designs. The apparatus is illustrated diagrammatically in Fig. 1.

* Received February 27, 1953, from the College of Pharmacy, University of Florida, Gainesville.
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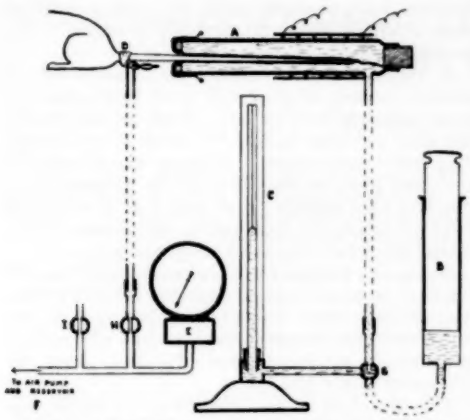

Fig. 1.—Blood pressure apparatus. A—plethysmograph; B—syringe; C—vertical capillary tube; D—pressure cuff; E—pressure gauge; F—air supply (not shown); G, H, I—valves.

PROCEDURE

The outer casing of a discarded glass water condenser was used for the barrel of the plethysmograph. A section 15 cm. long and 2.2 cm. in diameter with the side outlet near one end was selected and rubber stoppers fitted to each end.

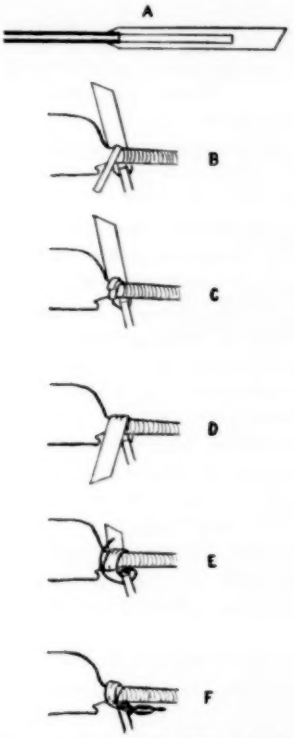

Fig. 2.—A—pressure cuff; B-E—steps in applying cuff; F—cuff applied and fastened with bull dog clamp.

The stopper in the end nearer the outlet tube closed off that end of the plethysmograph. A 1.3-cm. hole was bored in the other stopper and a metal ring fitted inside it. A condom rubber lining was inserted so that its closed end was anchored between the rubber stopper and the glass wall at the closed end of the plethysmograph. The open end of the condom was put through the hole in the other rubber stopper and the metal ring inserted to hold it in place. The excess portion of the lining was pulled back over the outside of the plethysmograph. Rubber weather-strip cement was used to seal the joints at each end. A short length of resistance wire was wound around the closed end of the plethysmograph and a piece of sheet asbestos fastened about it for insulation. A transformer or two 1.5-volt dry cell batteries were used to provide the small current necessary to heat the wire around the plethysmograph casing.

The remainder of the plethysmograph system consisted of an ordinary 20-cc. syringe, the vertical capillary tube connected to the side arm of the plethysmograph by 0.5-cm. polyethylene tubing, and a three-way valve. A metal needle adapter fitting into the three-way valve provided a joint for removing the plethysmograph from the rest of the system for replacement of the lining. The vertical capillary tube consisted of an 8-cm. section of 0.5-mm. glass capillary tubing joined to a section of larger bore tubing of like length. This was inserted into a discarded Bunsen burner base using a one-hole rubber stopper. Cement was used wherever necessary to make the system watertight. The Bunsen burner base made a good support of the vertical tube and provided a connection to the three-way valve through its side arm. A white card was fastened behind the capillary tube to facilitate reading of the water level. The system was filled with water colored with light green dye.

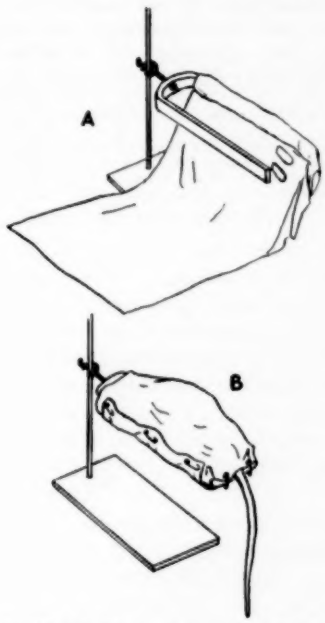

Fig. 3.—Rat holder. A—empty; B—in use.

The pressure system was made up of an air pressure supply, a pressure gauge, and a pressure cuff connected with polyethylene tubing and glass T-tubes. A 2-liter glass bottle with a rubber bulb air pump served as air supply and reservoir. An aneroid type pressure gauge calibrated from 20 to 300 mm. Hg was used to measure pressures. The cuff was that sold by Metro Industries for use with their Photoelectric-Tensiometer.[1] This cuff is only 5 mm. wide, and it must be carefully placed on the tail at the proper location or it will not completely stop tail circulation (6). Best results were obtained when the cuff was placed at the base of the tail just proximal to the point where the tail becomes scaly. This location and technique for putting on the cuffs are shown in Fig. 2.

The procedure for making a systolic blood pressure reading is as follows (references are to Fig. 1): Place pressure cuff on rat's tail. Turn three-way valve *G* so that plethysmograph *A* and syringe *B* are connected, and withdraw the plunger of the syringe so that water in the plethysmograph is evacuated. Insert rat's tail into the plethysmograph. Close valves *H* and *I* and pump air into reservoir *F* until the pressure seen on pressure gauge *E* is approximately 100 mm. Hg above the expected systolic pressure. Push the water in the syringe back into the plethysmograph and maintain a slight pressure on the plunger so that the tail is emptied of excess blood. While maintaining this pressure, open valve *H* to inflate the pressure cuff *D* which cuts off tail circulation. Release syringe plunger and turn three-way valve *G* so that plethysmograph *A* and vertical capillary tube *C* are connected. Allow water level in the capillary tube to come to equilibrium, manipulating valve *G* and the syringe, if necessary, to bring the water level near the bottom of the capillary. Open bleeder valve *I* slightly so that the pressure falls slowly. When the cuff pressure reaches the systolic pressure of the animal, blood enters the tail causing it to increase in volume since venous return is still blocked by the cuff. This volume change is communicated to capillary tube *C* by the water in the system and is seen there as a steady rise in the water level. When this rise occurs, immediately read the systolic pressure from pressure gauge *E*. The average of three readings within 10 mm. Hg is taken as the reading.

A convenient rat holder was constructed as follows: a strip of plastic 15 x 0.5 inches was cut from 0.25-inch sheet plastic. With the aid of heat the strip was bent into a "U" with sides 6 inches long and 2 inches apart. A clamp was fastened to the cross piece so that it could be attached to a ring stand for support. The end of an 8- x 15-inch piece of cloth was fastened to one prong of the plastic bar which was fixed horizontally.

In use, the rat was placed between the prongs and the cloth wrapped around both the rat and prongs and fastened with safety pins. Holes were cut in the cloth for the rat's hind legs since this seemed to make the rat less restless. Figure 3 shows the rat holder empty and in use.

The operation of the instrument is simple and speedy once the proper procedure is learned. Heating the plethysmograph casing is not necessary if the room temperature is moderately high. Perhaps the greatest difficulty and annoyance are encountered in the restless rat. However, once the operator is accustomed to the instrument, he can usually differentiate between changes in the capillary water level due to blood entering the tail and those due to actual movement of the rat.

When a series of blood pressures are to be taken on a single rat, as in studying a drug which has a rapid action on the blood pressure, single readings may be made as often as every two minutes. If a series of rats are to be run, requiring a change of rats in the holder and a change of pressure cuff from rat to rat, then readings may be obtained about once every four minutes if the operator is skilled and the rats are submissive to the required handling.

[1] Metro Industries, Chatham Phenix Building, 29-28 41st Avenue, Long Island City 1, N. Y. The Photoelectric-Tensiometer measures blood pressures by the leg-photoelectric cell method.

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Colorimetric Peroxide Test Method for Determining Vegetable Oil Stability*

By MILTON J. GOLDEN

Most of the methods recommended for determining vegetable oil stability have been found to be objectionable because of the time element involved, their impracticability, and their lack of sensitivity. A simple and reliable quantitative colorimetric peroxide test method is proposed for determining the peroxide content of vegetable oils. A definite and satisfactory correlation has been found to exist between the values of the colorimetric peroxide test method and those of the Swift stability method at 97.7°. This method can also be used to determine the peroxide content of oils at 125° with a saving of as much as 75 to 90 per cent of the time previously required by the Swift stability method.

In view of the fact that oils and fats as well as their derivatives form the basis of most of the cosmetic products manufactured today and that they readily undergo oxidation upon exposure to air, the use of antioxidants for their preservation in foods and cosmetic preparations has become increasingly widespread.

Some oils are known to resist atmospheric oxidation and have been found to have long induction periods before the onset of rancidity. This has been attributed in part to the presence of such natural inhibitors as tocopherols and other complex phenolic compounds that retard oxidation. Modern refining and processing tend to remove some of these natural inhibitors and, as a result, the refined oil is less resistant to oxidative rancidity than the crude oil.

This removal of natural inhibitors in the process of purification has necessitated the addition of synthetic inhibitors or antioxidants to improve stability.

Considerable attention also has been given to the evaluation of the numerous antioxygenic substances so recommended to determine their effectiveness in inhibiting and retarding rancidity.

In order to evaluate these compounds with respect to their antioxidant properties and to be able to detect actual and impending rancidity, several test methods have been proposed. Most of them, however, have been found to be either time-consuming, impractical, or unreproducible.

Such methods as the incubator test (1), the oxygen-absorption method (2–4), and the volatile aldehyde products test have been found to be objectionable because of the time element involved, their impracticability, and lack of sensitivity.

The quantitative determination of peroxides in oils and fats iodometrically is at the present time the most common method in use for determining and measuring stability (5). It has been assumed that this method and its modifications (6, 7) react quantitatively and may do so under somewhat rigid conditions. However, they do not always respond to fats and oils having different degrees of unsaturation and oxidation.

King, Roschen, and Irwin (8), recognizing the need for an improved method for determining the stability or keeping quality of oils and fats, introduced the so-called Swift stability test (active oxygen method) in which samples of oils and fats are aerated at 97.7°. The time required to obtain predetermined peroxide levels and other symptoms of rancidity is taken as a measure of their keeping quality. While this method has not found complete acceptance, it seems to be the predominant one in use today.

Several modifications of the Swift stability method have been recommended, particularly the catalytic action of metals (8) and use of higher temperatures to accelerate the decomposition of oils and fats (9–10). The results obtained from these two modifications varied considerably, and at elevated temperatures, the correlation was poor, probably because of the failure of the peroxides formed to be reduced by the HI and to the probable rearrangement and formation of peroxides different from those at 97.7°.

Recently, a new qualitative stability method, peroxide test method (11), has been suggested, eliminating to some extent most of the interferences inherent in the iodometric methods and making possible a considerable saving of time. A definite and satisfactory correlation has been reported to exist between the values obtained by the peroxide test method at 97.7° and 125° for vegetable oils and lard, and those by the active oxygen method at 97.7°.

In this test method, use is made of the ferrous thiocyanate reagent as a means of evaluating and comparing oils and fats with and without antioxidants. This reagent is not new. It has been known for several years and has made possible

* Received December 23, 1952, from the Research Laboratories, McKesson & Robbins, Inc., Bridgeport, Conn.

the development of such methods as the peroxide test for mineral oils (12) and petrolatums (13) and for the determination of the peroxide content of milk and fat (14).

Inasmuch as some investigators still prefer to express the extent of rancidity development and peroxide content of oils quantitatively, the peroxide test method has been modified to give such quantitative values. The recommended peroxide value of 80–100 milliequivalents per Kg. of oil has been chosen and used as the breaking point for vegetable oils.

EXPERIMENTAL

The facts and reactions as described in the peroxide test for mineral oil (12) and in the qualitative peroxide test method for determining oil and fat stability (11) were used in developing the following proposed method. In the peroxide test method (11), quantitative readings of the colored test solution in the Klett colorimeter were not possible because of the turbidity which developed upon mixing the oil phase with the test solution. To facilitate the reading of the oxidized ferrous thiocyanate reagent, a 25% ethyl ether in No. 10 mineral oil mixture was used. This ether-mineral oil combination seemed to clarify the test solution within a few seconds, making possible the reading of the test solution colorimetrically within a minute.

A volume of 0.1 cc. of oil, equivalent to approximately 0.092 Gm., is pipetted into a cork-stoppered special Klett test tube cell; 5 cc. of the ether-mineral oil mixture is then added to dissolve the oil, followed by 7 cc. of the colorless test solution. The air in the test tube is then replaced with carbon dioxide, stoppered with a new cork and shaken by hand for one minute. The lower test solution layer in the Klett tubes will vary in color from pink to dark red upon shaking and the amount of pink or red color formed is measured in the Klett colorimeter using the Klett filter No. 56.

Apparatus.—One-liter round-bottom flask and reflux condenser; constant temperature oil bath; Swift stability keeping test apparatus; Klett-Summerson photoelectric colorimeter with filter No. 56, having a mean transmission of 560 mμ, and special test tube cells, marked to indicate 5 and 12 cc. filling.

Materials.—Ferrous sulfate C. P.; ammonium thiocyanate C. P.; iron by hydrogen, Merck (reduced iron); iron wire No. 30 for standardization; acetone U. S. P.; 3% hydrogen peroxide solution; 25% ethyl ether (U. S. P.) in No. 10 mineral oil.

Preparation of Test Solution.—In a round-bottom, wide-mouthed flask, 2 Gm. of ferrous sulfate is dissolved in 100 cc. of distilled water containing 2 cc. concentrated sulfuric acid. Two hundred cubic centimeters of acetone and 100 cc. of a 2% aqueous solution of ammonium thiocyanate are then added to the above solution. Iron powder 0.2 Gm. and 6 inches of No. 30 iron wire are finally added to reduce any trace of ferric ion present. The resulting mixture is refluxed on a steam bath until the red color of the solution has disappeared. This reduction process takes about two hours, as it is necessary to continue the refluxing to insure lack of color.

Fresh solution should be prepared on the day of the test. It is possible, however, to preserve it in a colorless state by careful storage in a carbon dioxide atmosphere.

Since it is necessary to avoid any contact with air, a method has been devised to dispense this solution. A siphon is passed into the upper end of the reflux condenser through a two-hole cork stopper. The second hole is fitted with a glass stopcock, which serves as a vent. Carbon dioxide is admitted through the vent to inaugurate the flow of the solution, the pressure forcing the liquid into the siphon, at the end of which is attached a glass stopcock to withdraw the solution.

Procedure.—A volume of 0.1 cc. samples of oil, equivalent to approximately 0.092 Gm., is pipetted into cork-stoppered special Klett test tubes, marked to indicate 5- and 12-cc. filling; 5 cc. of the ethyl ether-mineral oil mixture is then added. The air in the flask is replaced with carbon dioxide, and the colorless test solution is then added to the 12 cc. mark. The test tube is stoppered again with the cork and shaken by hand for thirty seconds. Within a minute, the lower test solution layer, varying in color from pink to dark red, will separate from the oil mixture and be sparkling clear. The Klett test tube cells, containing sample and blank (without vegetable oil) are then placed within one minute in the Klett colorimeter, and the amount of pink or red color formed is measured using the Klett filter No. 56. The difference in scale readings between the sample and the blank gives the reading for the oxidation of the test solution by the peroxides present in approximately 0.092 Gm. of oil. The amount of peroxide oxygen contained in a kilogram of oil, expressed as mg./Kg., is then calculated accurately from the specific gravity of the oil at 25°. To minimize any error due to temperature changes, all solutions including the oil samples under study were pipetted at 25°.

Prior to the above described procedure, the Klett photoelectric colorimeter is adjusted to zero with water and then standardized with various concentrations of hydrogen peroxide by measuring the amount of color corresponding to the various quantities of peroxide. A freshly prepared 3% hydrogen peroxide solution is diluted 1–100, and its peroxide oxygen content is determined according to the U. S. P. assay method using 0.1 *N* $KMnO_4$ solution, each cubic centimeter of which is known to be the equivalent of 0.8 mg. of nascent oxygen. Very small volumes of this diluted peroxide solution, in the range of peroxide concentration of 1.2×10^{-4} to 1.2×10^{-3}%, are treated similarly as the unknown and the amount of pink or red color developed is measured in the colorimeter.

The peroxide concentrations are plotted against scale readings, and a standardization curve is obtained with increasing concentrations of peroxides. In the range of the peroxide concentrations suggested, the colored test solution has been found to obey Beer's law. The resulting graph facilitates the determination of the amount of peroxide by evaluating the concentration corresponding to the difference in the scale readings between the blank and sample.

DISCUSSION

To determine whether any correlation exists between the values obtained by the colorimetric peroxide test method and those of the Wheeler peroxide method (6) as modified by King and his associates (8) and the Swift stability method (8), the peroxide content of several samples of cottonseed oil and peanut oil were evaluated according to these methods.

TABLE I.—COMPARISON OF COLORIMETRIC PEROXIDE TEST METHOD VALUES WITH THOSE OF THE MODIFIED WHEELER PEROXIDE METHOD AT 25° C.

Sample	Peroxide Test, Mg. Peroxide/Kg. Oil	Modified Wheeler Peroxide Method Millimoles Peroxide/Kg. Oil
Cottonseed Oil		
(*A*)	18	19
(*B*)	24.2	24
(*C*)	29	27
Peanut Oil		
(*A*)	8	9.2
(*B*)	21	19
(*C*)	24	22

Tables I and II show our findings at 25, 97.7, and 125°, when tested according to the suggested colorimetric peroxide test method, the modified Wheeler peroxide method and the Swift stability method of King and his associates. The values of the colorimetric peroxide test method at 25 and 97.7°, expressed as mg. of peroxides per Kg. of oil seem to approximate fairly closely the values obtained by the modified Wheeler peroxide method. Moreover, the times required to reach the reported peroxide levels at 97.7° by these two methods are the same. At 125°, both the modified Wheeler and Swift stability methods gave unreproducible values with variations as great as 25% in both methods. The colorimetric peroxide test method at 125°, however, gave reproducible values with a considerable saving of time.

The values reported in Tables I and II represent the average of at least three separate determinations deviating not more than ±2%.

It is apparent from our findings that the recommended peroxide value of 80–100 millimoles peroxide per kilogram oil as the breaking point for oils can be used with the colorimetric peroxide test method when carried out at 125°, the temperature reported to make possible a saving of as much as 75–90% of the time previously required by the Swift stability method. This suggested colorimetric peroxide test method will eliminate to a great extent the interfering factors and shortcomings of both the modified Wheeler and Swift stability methods at temperatures over 97.7°.

TABLE II.—COMPARISON OF COLORIMETRIC PEROXIDE TEST METHOD VALUES WITH THOSE OF THE MODIFIED WHEELER PEROXIDE METHOD AND THE SWIFT STABILITY METHOD AT 97.7 AND 125° C., BASED ON THE PEROXIDE VALUE OF 80–100 MILLIMOLES PEROXIDE PER KILOGRAM OF OIL

Sample	Peroxide Test, Mg./Kg., at 97.7° C.	Modified Wheeler Peroxide Method, Millimoles/Kg., at 97.7° C.	Time Required, hr. Peroxide Test 97.7° C.	Peroxide Test 125° C.	Swift Stability Method, 97.7° C	Swift Stability Method, 125° C.
Cottonseed Oil	80	79	5	1 1/4	5	Unreproducible values
Peanut Oil	95.5	97	3	5/6	3	Unreproducible values

SUMMARY

1. A simple and reliable quantitative colorimetric peroxide test method is proposed for determining the peroxide content of oils.

2. A definite and satisfactory correlation has been found to exist between the values of the colorimetric peroxide test method and those of the modified Wheeler peroxide and the Swift stability methods at 25 and 97.7°.

3. This suggested peroxide test method can be used to determine quantitatively the peroxide content of oils at 125° with a saving of as much as 75–90% of the time previously required by the Swift stability method; it also eliminates to a great extent the interfering factors and shortcomings of the modified Wheeler and Swift stability methods at temperatures over 97.7°.

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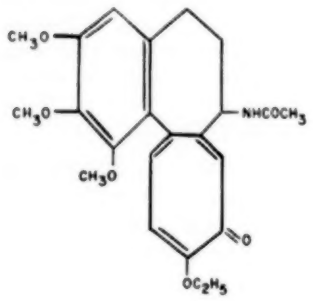
Countercurrent Distribution of Colchicine and some Derivatives*

By S. D. BAILEY,† R. M. HOROWITZ,‡ and H. L. DINSMORE§

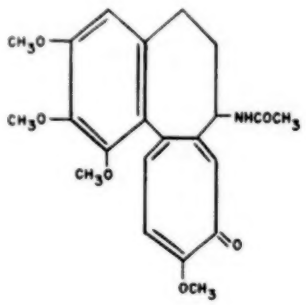
A method for the determination of the composition of a binary mixture based on partition coefficients is reported. Where a significant difference exists in the partition coefficients of the two components for a given solvent system, an equation is presented which permits composition analyses where other methods may not be applicable. The method has been applied to the determination of colchicine present in a sample of colchiceine ethyl ether as a starting material contaminant. Confirmation of the method is shown by a nine-plate countercurrent distribution of a mixture of colchiceine ethyl ether and colchicine based on the composition analyses obtained using the equation developed.

THE SYNTHESIS of colchiceine ethyl ether, I by two different methods, was recently reported (4) from these Laboratories. In one case colchiceine was ethylated with ethyl sulfate and in the second, colchiceine methyl ether (colchicine), II, was transetherified to the ethyl ether I. The product obtained by the latter method gave unsatisfactory carbon-hydrogen analyses and the presence of extraneous bands in its infrared absorption spectrum suggested contamination by the starting material, II. In order to determine the amount and the nature of the contaminant, a nine-plate countercurrent distribution analysis was conducted using the apparatus described by Raymond (7). A third compound isocolchicine, III, was also studied because of the close structural relationship to compounds I and II. In the estimation of the composition of the binary mixture obtained in method *two*, an equation was applied from which the mole fractions of a qualitatively known binary mixture may be determined in terms of the partition coefficients of the mixture and of the pure components. In this study, no attempt has been made to separate the mixture by the use of higher numbers of transfers which have been described by Craig (1) and others in the field. This has been accepted as standard procedure where more complex mixtures are handled. For simple binary mixtures of known qualitative composition, the relationship of partition coefficients of the known components and the mixture permit analysis by the use of single extraction techniques or by countercurrent distribution. In the case of single extractions the method has been used in the determination of the hydrolysis rates of esters of benzylpenicillin (3, 5). A similar method was employed by Wright and Grove (6) for the estimation of penicillin K in commercial penicillin.



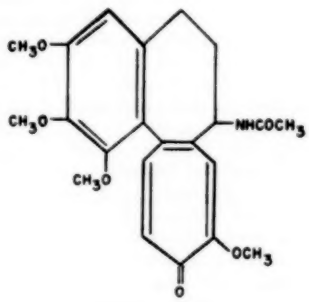
I

COLCHICEINE ETHYL ETHER



II

COLCHICEINE METHYL ETHER (COLCHICINE)



III

ISOCOLCHICINE

* Received November 5, 1952, from the Research Division of the Smith, Kline and French Laboratories, Philadelphia, Pennsylvania.

The investigation was supported in part by a research grant to Glenn E. Ullyot from the National Cancer Institute; R. M. Horowitz, Research Associate.

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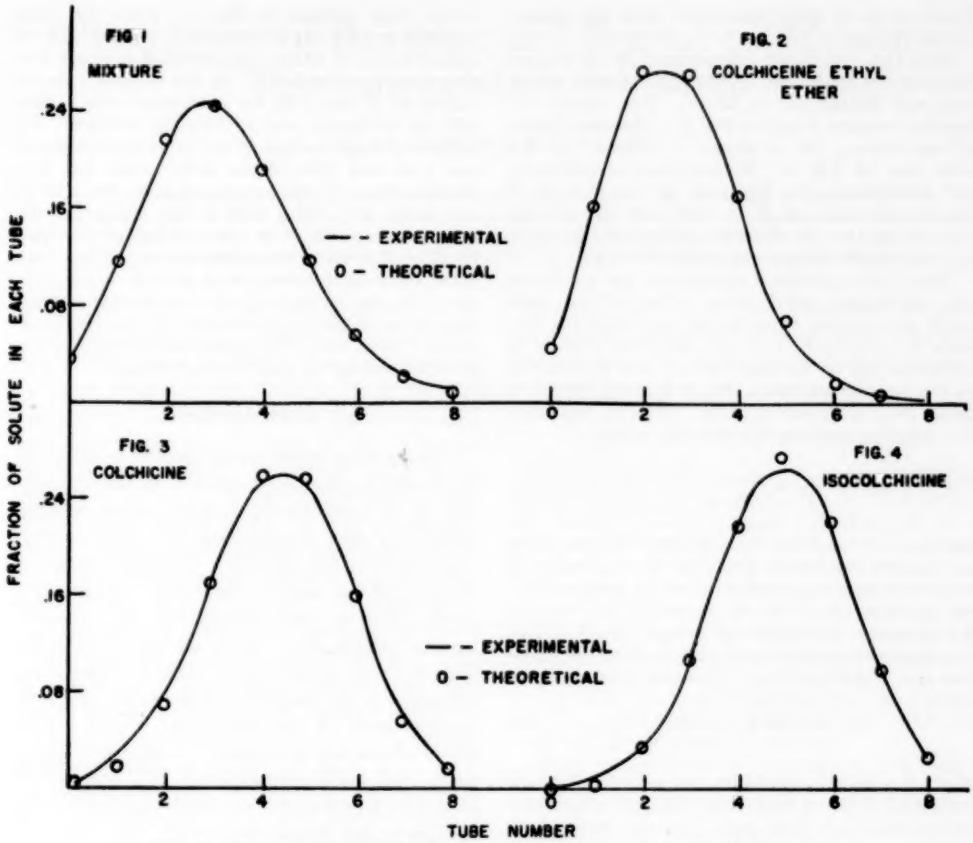

Fig. 1.—Counter-current distribution of colchiceine ethyl ether (mixture).
Fig. 2.—Counter-current distribution of colchiceine ethyl ether.
Fig. 3.—Counter-current distribution of colchicine.
Fig. 4.—Counter-current distribution of isocolchicine.

EXPERIMENTAL

The countercurrent distribution was carried out using equal volumes of the immiscible solvent system-ethyl acetate/water. With the material dissolved in the lower layer of the first tube, successive transfers and equilibration of this layer with fresh upper layer, distributed the material throughout the nine tubes as shown in column 2 of Table I. The combined layers of each tube were made homogeneous by the addition of 95% ethanol and were assayed by measuring the absorption band in the ultraviolet at 350 mμ, using a Cary recording spectrophotometer. The data shown in the table are fractions of the total absorbance for the combined layers of the nine plates. These fractions were then plotted against the tube numbers to give the distribution curve of Fig. 1. No satisfactory theoretical distribution values, based on a single partition coefficient, could be made to fit the curve.

The preparation of I by ethylating colchiceine (method *one*) provided material giving the correct carbon-hydrogen analysis and showing no spectral evidence of contamination with starting material. This sample, when distributed exactly as outlined above, gave the distribution data shown in column 3, Table I, and the distribution curve of Fig. 2. Theoretical values calculated for a partition coefficient of 2.20 (indicated by circles, Fig. 2) were

TABLE I.—COUNTERCURRENT DISTRIBUTION DATA

	Fraction Present in Each Tube[a]			
Amount Distributed → / Tube	Mixture 16.3 Mg.	Colchiceine Ethyl Ether (I) 5.62 Mg.	Colchicine (II) 19.1 Mg.	Isocolchicine (III) 21.9 Mg.
0	0.034	0.041	0.004	0.002
1	0.112	0.161	0.030	0.011
2	0.219	0.270	0.073	0.033
3	0.246	0.261	0.164	0.099
4	0.196	0.168	0.254	0.227
5	0.118	0.067	0.249	0.268
6	0.048	0.021	0.155	0.231
7	0.018	0.005	0.058	0.107
8	0.008	0.006	0.014	0.023

[a] Expressed as fractions of the total absorbance at 350 mμ.

found to be in good agreement with the experimental values.

Since the most likely contaminant in the impure material was colchicine, II, a sample of pure colchicine was distributed as above. The results are shown in column 4 and by Fig. 3. The distribution of isocolchicine, III, is shown in column 5 of the table and by Fig. 4. Homogeneity of colchicine and isocolchicine was indicated by the close fit of theoretical values of $K = 0.90$ and $K = 0.63$, respectively, to the experimental curves in which the theoretical values are denoted by circles.

Using these partition coefficients for colchicine and colchiceine ethyl ether (obtained by both single distribution experiments and from the formula $N = n(K)/K + 1$ (2); the mole fraction of colchicine (or of the ethyl ether) was determined on the assumption that a binary mixture existed of these two components. The following equation was found useful for this determination:

$$X_A = 1 - X_B = \frac{(K_X - K_B)(K_A + 1)}{(K_A - K_B)(K_X + 1)} \quad (1)$$

where X_A is the total mole fraction of component A, X_B the total mole fraction of component B, K_X is the partition coefficient of the mixture, K_A the partition coefficient of component A, and K_B the partition coefficient of component B. This equation may be developed readily from the partition law, $x'_A/x''_A = K_A$, by putting it in the form

$$x'_A = X_A \frac{K_A}{K_A + 1} \quad (2)$$

where x'_A and x''_A represent the mole fractions of compound A in the upper and lower phases, respectively, and X_A is their sum. For the lower phase this becomes

$$x''_A = X_A \frac{1}{K_A + 1} \quad (3)$$

Since $K_X = (x'_A + x'_B)/(x''_A + x''_B)$ it follows that

$$K_x = \frac{X_A \frac{K_A}{K_A + 1} + (1 - X_A) \frac{K_B}{K_B + 1}}{X_A \frac{1}{K_A + 1} + (1 - X_A) \frac{1}{K_B + 1}} \quad (4)$$

which then reduces to Eq. 1. From the three partition coefficients determined experimentally the mole fraction of either component of a binary mixture may be calculated. In the present case the values of K are 2.20 for colchiceine ethyl ether, 0.90 for colchicine, and 1.70 for the mixture. The calculated mole fraction of the impurity (colchicine) was 0.27 and that of the ethyl ether was 0.73. Confirmation of this composition of the mixture was made by adding 0.73 of the theoretical distribution values for $K = 2.20$ and 0.27 of the values for $K = 0.90$ for a nine-plate system. These combined theoretical values when plotted in Fig. 1, as shown by the circles, indicate a reasonable explanation of the observed distribution of the mixture in terms of these two components, since there is a close fit of theoretical to experimental values.

CONCLUSION

The equation found would seem to be generally applicable to the determination of the composition of a qualitatively known binary mixture where the two components are independently distributed. The partition coefficients may be determined by simple distribution employing a single plate, or, more accurately, by a countercurrent distribution. It is obvious that it applies most accurately where there is a significant difference in the partition coefficients of the components and at concentrations where solute-solute effects are minimized. With these limitations, and where separation is not required, it should be found useful in connection with countercurrent and simple distribution data of binary mixtures.

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The Effect of Banthine and Prantal on Human Thermoregulatory Sweating*

By MORTON J. RODMAN†

Thermally stimulated sweating was considerably reduced by all doses of Banthine employed in this study. Prantal was less effective than Banthine but could be used in larger doses, due to its comparative freedom from annoying side effects. Atropine, in relatively small doses, had a high index of anhidrotic effectiveness and safety.

THE RESULTS of an ever increasing number of clinical papers have indicated the effectiveness of the newer synthetic spasmolytic drugs, Banthine bromide[1] and Prantal Methylsulfate[2] as adjuncts in the management of peptic ulcer and other gastrointestinal disorders (1–6). The anticholinergic nature of this action has lead inevitably to an interest in the possible effectiveness of these drugs in the treatment of hyperhidrosis, a rather common and distressing condition, which frequently proves resistant to even the most heroic therapy. Recent reports (7–9) of the results of administration of these drugs to small groups of hidrose patients have suggested that both may have a place in controlling excessive sweating. Zupko and Prokop (10) have recently presented the first comparative evaluation of the two drugs in the treatment of hyperhidrosis.

It was the purpose of this study to determine the extent to which various dose levels of each of these drugs inhibits heat-induced sweating in normal human subjects and to compare the effectiveness and toxicity of these drugs with that of atropine, a traditional anhidrotic agent, which was recently studied in a similar manner (11).

A larger goal of this series of studies is the validation of a quantitative laboratory method of screening drugs for anhidrotic activity. While the results of studies such as this are obviously not directly transferable to the treatment of hyperhidrosis of nervous origin, it is felt that one may justifiably draw certain inferences as to the relative therapeutic indices of the agents employed. At the same time, a comparison of the results of this work with those of the clinical reports to follow may serve to establish whether the techniques herein described can be applied routinely in the laboratory to determine the relative sweat-reducing potency of new compounds.

METHODS AND PROCEDURES

The extent to which sweating is lessened by prior administration of various dose levels of Banthine and Prantal was determined quantitatively in a group of nine young men exposed in a constant temperature and humidity chamber for one hour daily during a period of several weeks to a temperature of 104° F. D. B./87° F. W. B. (approximately 50% relative humidity). The amount of sweat obtained after administration of Banthine and Prantal was compared with that which was collected under the same conditions after administration of a placebo or of atropine 1.2 mg., which was employed as a standard of reference.

Sweat was collected by absorption into highly absorbent cotton pads contained in a capsule of transparent plastic (11). At the beginning of the test period, the identifying cover was removed and stored in a desiccator. The capsule was then inverted over a circular area of abdominal skin 93 mm. in diameter between the xiphoid and the umbilicus, taped firmly to the skin, and further secured by a wide abdominal binder made of gauze and containing layers of absorbent cotton to take up excess moisture from the skin around the test area. At the conclusion of the one-hour test period, during which the subject reclined or sat quietly in the chamber, the capsule was removed from the skin with speed and care, capped with its tightly fitted cover, and immediately weighed on a precision balance. The difference in weight between the capsule at this time and its weight just prior to application represented the sweat secreted in the area during the hour of heat.

In a number of test periods, sweat gland activity was visualized simultaneously on a part of the abdominal skin adjacent to that covered by the capsule. This area was painted with a 2% alcoholic solution of ferric chloride, which was allowed to dry; the area was then sprayed with finely powdered potassium ferrocyanide. Sweating resulted in the appearance of blue-black drops at the site of functioning glands, due to the formation of Prussian Blue in the presence of moisture.

Pulse rate, body temperature, and physical condition of each subject were observed immediately before and after every test period. In addition, subjective responses for the period of the test and for the six to eight hours following, were fully reported and recorded in order to determine the

* Received January 14, 1953, from Rutgers University College of Pharmacy, Newark, New Jersey.

Presented to the Pharmacy Subsection, American Association for the Advancement of Science, St. Louis meeting, December 29, 1952.

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This study was partially supported by funds furnished by Rutgers University, The Schering Corporation, Bloomfield, N. J., and G. D. Searle and Company, Chicago, Ill.

The author wishes to thank Doctor George Babcock, Jr., of the Schering Corporation for his kind interest and for supplying Prantal for this study. Thanks are due also to Doctor J. William Crosson of G. D. Searle and Company for providing the Banthine used in this study.

[1] β-diethylmethylaminoethyl-9-xanthenecarboxylate bromide.

[2] N,N-dimethyl-4-piperidylidene-1,1-diphenylmethane methylsulfate.

duration and intensity of action of each of the various doses used.

The subjects were carefully instructed in a procedural routine intended to control those factors which are believed to produce variability in sweat tests that extend over a period of many weeks (12, 13). A later statistical analysis of the data from the control tests indicated with a high degree of significance that such variables were largely under control. This may be due in large part to the intelligent cooperation of the group, which was comprised of medical school senior students and advanced graduate students in medical sciences.

All of the various doses of the several drugs and placebos were distributed in a thoroughly randomized manner over the weeks of the experimental period. Each of the three doses of Banthine employed (100 mg., 200 mg., and 300 mg.) and of Prantal (200 mg., 400 mg., and 600 mg.) was administered four times in the case of nearly every subject; atropine 1.2 mg. was given three times to each subject, and each received a placebo on eight occasions, in order to determine rates of sweating under control conditions. The atropine dose and each of the doses of Banthine (and its placebos) were administered in capsules between forty-five to sixty minutes before the subject entered the preheated chamber. Capsules of Prantal (and its placebos) were generally swallowed between ninety to one hundred and ten minutes prior to the test period, due to the slower rate at which oral doses of Prantal reach peak effectiveness (14).

RESULTS

Effect of the Drugs on Thermal Sweating.—Table I summarizes the data obtained in over 300 one-hour trials and indicates the mean amounts of sweating for each subject and for the whole group under control conditions and after each of the various doses of the drugs.

As is usual in such studies, a wide range of individual variation was apparent in the amounts of sweat collected from the subjects in the control tests. A statistical analysis of the data from this comparatively small sample suggested a positive correlation of sweating with weight and height.

The minimum doses employed in this study were chosen after preliminary trials which indicated that smaller doses of Banthine (50 mg.) and of Prantal (100 mg.) were only slightly effective and rather variable in their ability to reduce sweating induced by high heat. Prantal 600 mg. was selected as a maximum dose for that drug after tests in which single doses of 800 mg. and 1,000 mg. of Prantal exerted no significantly greater activity than did 600 mg. Banthine 300 mg. was judged the maximum single dose of that drug which could be administered routinely without causing severe discomfort. Atropine 1.2 mg. was used, because earlier work in this laboratory (11) had suggested that this dose of atropine had a high index of effectiveness and safety.

The response to Banthine in all doses was more uniform than that to Prantal and atropine, and the effect of any dose of Banthine on sweating was generally more readily predictable than that of either of the other drugs (Table II). In all subjects, Banthine was considerably more effective than Prantal

TABLE I.—MEAN SWEAT RATES FOR EACH OF NINE SUBJECTS AND FOR GROUP AS A WHOLE (GRAMS/1 HOUR/93 SQ. IN. DIAM. CAPS)

No.	Subject Age	Wt. Lb.	Ht. In.	Control	Atropine, 1.2 Mg.	Prantal 200 Mg.	Prantal 400 Mg.	Prantal 600 Mg.	Banthine 100 Mg.	Banthine 200 Mg.	Banthine 300 Mg.
1.	34	175	72	1.146	0.539 (−53%)	0.892 (−22%)	0.794 (−31%)	0.517 (−55%)	0.772 (−33%)	0.508 (−56%)	0.397 (−65%)
2.	26	176	70	1.025	0.689 (−33%)	0.991 (− 3%)	0.741 (−28%)	0.689 (−33%)	0.657 (−35%)	0.449 (−56%)	0.158 (−85%)
3.	24	180	70	0.971	0.410 (−58%)	0.644 (−34%)	0.548 (−44%)	0.457 (−53%)	0.437 (−55%)	0.365 (−65%)	0.272 (−72%)
4.	30	158	67	0.685	0.418 (−39%)	0.520 (−24%)	0.495 (−28%)	0.328 (−52%)	0.369 (−46%)	0.223 (−67%)	0.180 (−74%)
5.	27	175	69.5	1.240	0.483 (−61%)	0.953 (−23%)	0.937 (−24%)	0.704 (−43%)	0.984 (−21%)	0.658 (−47%)	0.257 (−79%)
6.	23	230	72	1.726	0.718 (−58%)	1.256 (−27%)	0.957 (−44%)	1.017 (−41%)	0.922 (−40%)	0.684 (−60%)	0.656 (−62%)
7.	25	186	71	1.061	0.591 (−44%)	0.856 (−19%)	0.730 (−31%)	0.411 (−61%)	0.657 (−38%)	0.478 (−55%)	0.378 (−64%)
8.	27	193	72	1.416	0.931 (−34%)	0.925 (−35%)	0.778 (−45%)	0.629 (−56%)	0.578 (−59%)	0.469 (−67%)	0.452 (−68%)
9.	25	192	68.5	0.793	0.548 (−31%)	0.676 (−15%)	0.548 (−31%)	0.462 (−45%)	0.556 (−30%)	0.449 (−45%)	0.253 (−68%)
Mean				1.118	0.592 (−47%)	0.857 (−23%)	0.730 (−36%)	0.517 (−48%)	0.659 (−41%)	0.476 (−57%)	0.334 (−70%)

TABLE II.—GROUP MEANS. EFFECTS OF SEVERAL DOSE LEVELS OF BANTHINE, PRANTAL, AND ATROPINE UPON THERMAL SWEATING, PULSE RATE, AND BODY TEMPERATURE

Drug	Mean Sweat, Gm./93 Mm. Caps	Mean Pulse Rate Before Heat	Mean Pulse Rate After Heat, 1 Hour	Mean Body Temp. Before Heat, ° F	Mean Body Temp. After Heat, 1 Hour ° F
Placebo	1.118	86	93	98.4	99.0
Atropine 1.2 mg.	0.592 (−47%)	80	108	98.4	99.1
Prantal 200 mg.	0.857 (−23%)	89	95	98.4	99.0
Prantal 400 mg.	0.720 (−36%)	95	101	98.4	99.1
Prantal 600 mg.	0.517 (−48%)	95	107	98.4	99.1
Banthine 100 mg.	0.659 (−41%)	88	102	98.4	99.1
Banthine 200 mg.	0.476 (−57%)	95	113	98.5	99.3
Banthine 300 mg.	0.334 (−70%)	101	122	98.5	99.6

in anhidrotic activity. An examination of equi-effective doses suggests that Banthine was generally between three and six times as potent as Prantal in this respect, and, that, of the doses used, Banthine 100 mg., Prantal 400–600 mg., and atropine 1.2 mg. most often had about the same degree of effectiveness—a 40–50% reduction in sweating accompanied by some degree of xerostomia. Increments in the dose of Prantal resulted in a reduction in sweating somewhat more steep than that brought about by raising the dose of Banthine proportionately. However, as had been the case in an earlier study of atropine (11), the effectiveness of Prantal seemed to level off at a reduction in sweating of about 50%. On the other hand, when the dose of Banthine was raised to 300 mg., mean sweat ing was reduced from normal by about 70% aud, in some individual trials, by as much as 85–90%.

The colorimetric tests performed simultaneously revealed a number of interesting differences between normal patterns of sweat gland activity and those which developed under the influence of atropine, Banthine, and the largest dose of Prantal (600 mg.). These results are to be reported in detail elsewhere. However, the following observations can be noted at this time: in the untreated group, the onset of sweat gland activity varied widely (one and one-half to twelve minutes after exposure to heat), but in almost every case, this onset of activity occurred earlier in this group than in that which had received effective doses of the anticholinergic drugs. During the first fifteen minutes of heat, there appeared to be no difference between the two groups in the output of sweat from such glands as were activated. In the second fifteen-minute period both the number of active glands and the output per gland increased steadily in the placebo group; in the treated group, the number of active glands also increased but to a lesser extent, and the output per gland remained low. In the control group, there was no further change during the remaining thirty minutes of the test period in either the number of functioning glands or in the output per gland, except for intermittent cyclic increases and decreases in the activity of various individual glands. During this final period, the number of functioning glands of the treated group remained less than in the control group. However, unlike the latter group in which sweating had leveled off at a maximal "plateau" rate, the amount of sweat secreted by the treated group increased during this period, due to an increase in the output of many of the functioning glands. Thus, the nature of sweat gland activity after oral doses of anticholinergic drugs appears to be essentially the same as normal activity, so that any difference is one of degree rather than of kind. There was no indication of the "complete paralysis" of the sweat glands which has been described in animal experiments after intravenous injections of atropine.

TABLE III.—COMPARISON OF SIDE EFFECTS CAUSED BY DRUGS AND HEAT. PERCENTAGE OF TRIALS IN WHICH EFFECT OCCURRED

	Atropine, 1.2 Mg.	Prantal 200 Mg.	Prantal 400 Mg.	Prantal 600 Mg.	Banthine 100 Mg.	Banthine 200 Mg.	Banthine 300 Mg.
No. of Trials	27	33	35	35	35	35	32
Type of Effect							
1. Xerostomia							
Slight	33.3	9.1	48.6	42.9	34.3	8.6	...
Moderate	22.2	...	2.8	17.1	40.0	34.3	12.5
Severe	11.1	...	...	8.6	25.7	57.1	87.5
Total	66.6	9.1	51.4	68.6	100.0	100.0	100.0
2. Gastrointestinal							
Constipation	...	...	...	...	2.8	5.7	15.6
Heartburn	...	...	...	...	...	2.8	6.2
Diarrhea	...	...	2.8	2.8	...	...	...
3. Cycloplegia and Mydriasis (Blurring of Vision)	3.7	...	8.6	5.7	8.6	5.7	31.3
4. Headache	...	...	...	...	11.4	14.3	43.7
5. Dysuria	...	...	...	...	...	5.7	21.9
6. Paresthesia	3.7	3.0	2.8	2.8	2.8	5.7	21.9
7. Other	Flushing 11.1	...	...	Sedation 2.8	...	Sedation 2.8	Sedation 9.4
	...	...	...	...	...	Flushing 14.3	Flushing 31.3

Side Effects.—Table III compares the side effects caused by atropine and by the various doses of Prantal and Banthine. Oral doses of Prantal proved to be quite free of annoying side effects, even in the largest dose routinely employed. In this dose (600 mg.), which reduced sweating nearly 50%, the only common side action was a generally mild xerostoma. Even in single doses as high as 1,000 mg., there was no very annoying reaction to Prantal, though the dryness of the mouth was more apparent and blurring of vision more pronounced The side effects of atropine 1.2 mg., an equieffective dose, were very similar to those of Prantal 600 mg. in kind and degree. The action of Banthine, on the other hand, was accompanied by various annoying side reactions. With even the smallest dose of Banthine (100 mg.), every subject reported some degree of xerostomia, and in the majority of cases, the dryness of the mouth was classified as moderate to severe. When larger doses of Banthine were used, this drying effect was intensified, and complaints of a bitter taste, hoarseness, and thirst were common. Headache and blurring of vision were also frequent, especially with the largest dose of Banthine (300 mg.). Some of the discomforting results of Banthine at this dose level were due not to the direct effects of the drug alone, but probably reflected some degree of heat stroke caused by interference with sweating, the chief mechanism for heat loss when environmental temperatures rise to 88–90° F. It may be noted, at this point, that according to most subjects, Banthine 300 mg. reduced sweating for as long as six to eight hours, even in the unusually humid heat of the past summer in Washington, D. C. (Table IV). This action was not a useful one, however. On the contrary, the prolonged reduction in sweating brought about by this dose of Banthine seemed to add to the discomfort. The smaller doses of Banthine were also reported to reduce sweating beyond the test period —Banthine 200 mg. for about two to four hours after the chamber tests and Banthine 100 mg. for about one or two hours. The latter dose and equieffective doses of Prantal (600 mg.) and of atropine (1.2 mg.) were the only ones that lessened sweating to any extent and for any length of time during the afternoon heat of the city, without at the same time, causing increased discomfort and illness.

An interesting effect reported occasionally by some subjects with every dose level of all three drugs, but not with placebos, was a form of paresthesia, which does not appear to have been previously noted in the literature. This was reported by some subjects to be a peculiar sensation of coolness in the lower legs, thighs, and buttocks, and by others as a warm, tingling sensation in the same areas. The fact that these sensations occurred about one hour after administration of the drug and prior to exposure of the subject to high heat indicates that this reaction is due to the drugs alone and not to a combination of the drugs with heat.

Effects on Body Temperature and Pulse Rates.—The effects of heat and of a combination of heat and the various drugs on body temperature and pulse rates are shown in Table II. In no case did any drug alone bring about any significant change in body temperature, nor did any drug, except Banthine 300 mg. together with exposure to heat, cause a rise in body temperature significantly larger than was brought about by an hour of heat in control tests.

All of the drugs had some effect on the cardiac mechanism. With the exception of atropine 1.2 mg. which produced a slowing of the heart rate in most cases by central vagal stimulation, most of the anticholinergic agents caused some degree of cardiac acceleration due to peripheral vagal depression. The increase in heart rate was intensified by exposure to heat, but only in the case of the two higher doses of Banthine did the pulse rate ever attain unusually high levels—140–160 b. p. m. in some instances—and even with these doses such reactions were not common.

DISCUSSION

The potent anhidrotic action of Banthine is evident in all the doses used under the experimental conditions. Banthine 100 mg. is capable of bringing about a significant reduction in thermal sweating with but comparatively few annoying side effects, and, thus, this dose may have clinical usefulness in the treatment of hyperhidrosis. Although the annoying side effects which accompany administration of the larger doses of Banthine seem to obviate their common use, clinically, it is of interest to note that these reactions are not of a type that tends to antagonize the peripheral depression of the sweat glands by Banthine. Thus, in spite of the occurrence of such side effects, Banthine 300 mg. brings about a reduction of sweating as high as 85% in some cases. This is a reduction considerably greater than occurred in a previous study (11) of atropine in oral doses so high as to cause toxic symptoms much more severe than those caused by Banthine. It was suggested in the earlier study that the anhidrotic effectiveness of high oral doses of atropine was self limited due to complex effects on the central nervous system, body temperature, and pulse rate of a kind that tend to antagonize the peripheral depression of the sweat glands by the drug. Such an antagonism of peripheral depressant action does not seem to occur to any great extent with the higher doses of Banthine. Although such doses bring about some increase in body temperature and pulse rate, these are not generally so high as to set up conditions that are favorable to sweating and consequently, do not tend to neutralize the peripheral depressant action of high doses of Banthine on the sweat glands.

Another factor in the anhidrotic effectiveness of Banthine may be its ability to inhibit the transmission of impulses at autonomic ganglia as well as at the sympathetic cholinergic neuroeffectors in the sweat glands themselves. Evidence has recently been presented (15–17) which suggests that there is an adrenergic component in the innervation of some sweat glands. Ganglionic blockage by Banthine could lessen the number of impulses passing to such epinephrine-sensitive glands. The blocking action of atropine, which occurs, in usual doses, only at postganglionic cholinergic neuroeffectors, would, on the other hand, be ineffective against such adrenergic impulses. In any case, this ganglionic depressant action of Banthine combined with the action of the drug at the neuroeffector, is very prob-

TABLE IV.—CLIMATOLOGICAL DATA WASHINGTON, D. C. (JUNE AND JULY, 1952)

Month	Maximum Temperatures: Daily Ave. Max. Temp., °F	Maximum Temperatures: No. of Days Max., 85° F. or Above	Maximum Temperatures: No. of Days Max., 90° F or Above	Psychrometric Data: Time: 1300 Hours (1:00 P. M.): Dry Bulb, °F	Time: 1300 Hours (1:00 P. M.): Wet Bulb, °F	Time: 1300 Hours (1:00 P. M.): Rel. Hum., %	Time: 1900 Hours (7:00 P. M.): Dry Bulb, °F	Time: 1900 Hours (7:00 P. M.): Wet Bulb, °F	Time: 1900 Hours (7:00 P. M.): Rel. Hum., %
June	86.7	18	13	83	69	51	78	68	61
July	89.6	27	16	87	73	50	83	72	60

ably another factor adding to the effectiveness of Banthine in reducing sweating.

Under the conditions of this study, Banthine appeared to be considerably more effective than Prantal in reducing thermal sweating. However, Zupko and Prokop (10) conclude that Prantal checks hyperhidrosis with only slightly less effectiveness than does Banthine. This more favorable comparative evaluation of Prantal by these workers may be due to differences in the type of subjects studied and in the techniques employed for eliciting and measuring sweating. In any case, one can agree with their conclusion that Prantal causes considerably fewer and milder side effects than does Banthine. This comparative freedom from side effects may permit the use of Prantal in doses large enough to equal Banthine in clinical effectiveness in many instances. It may be added here that neither of the newer drugs shows any distinct clinical advantage over atropine, which, in the dose used in this study, showed a high index of effectiveness and safety.

Oral doses of Prantal have a slower onset and less intensity of action than do either Banthine or atropine. This together with the leveling off in effectiveness of Prantal at doses above 600 mg. suggested that the drug was either comparatively slowly absorbed from the gastrointestinal tract or rather rapidly detoxified, so that sufficiently high concentrations of the drug at the sweat gland neuroeffectors were difficult to attain.

In order to determine whether these factors account for the relatively weak anhidrotic action of Prantal when it is used in doses that are usually effective in the treatment of various gastrointestinal conditions, a series of tests were made in which each subject, on at least two occasions, received Prantal 0.5 mg./Kg. by intramuscular injection. In every subject the results were substantially the same: within five to ten minutes after injection, the skin became hot, dry and flushed, moderate to severe xerostomia and blurring of vision occurred, and the heart rate increased to about 120–140 b. p. m. During the first twenty to thirty minutes of exposure to heat, the heart rate continued to be high and irregular, and sweating was very slight. However, the effects of the injection appeared to pass off rather rapidly during the last thirty to forty minutes of exposure to heat and moderate sweating occurred during this period. The final result of such intramuscular administration of Prantal was a reduction of sweating of only about 30% during the one-hour exposure to heat. This was accompanied by numerous side effects which do not occur with equally effective oral doses of the drug.

It may be concluded that the anhidrotic effectiveness of Prantal by oral administration is probably lessented by a relatively slow rate of absorption from the gastrointestinal tract and a relatively high rate of detoxication by the body tissues.

SUMMARY AND CONCLUSIONS

1. Oral administration of Banthine 100 mg., Prantal 400–600 mg., and atropine 1.2 mg., all reduced thermally stimulated sweating by about 40–50% in most subjects with a minimum of side effects.

2. Thermal sweating is not reduced significantly beyond this level (50%) by oral administration of larger doses of atropine and Prantal. Sweating is reduced by at least 70%, however, when the oral dose of Banthine is raised to 300 mg.

3. Possible mechanisms are suggested to account for the limited effectiveness of orally administered atropine and Prantal and for the greater potency of Banthine.

4. Prantal causes relatively few side effects, even at higher dose levels. Side reactions are more frequent and more varied with Banthine, and at higher dose levels, especially in a warm environment, the effects of Banthine may produce considerable discomfort.

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Toxicity Studies of a Nonionic Detergent*

By KATHLEENE M. SWEENEY and KURT A. OSTER

Single and repeated dose toxicity studies were performed with a nonionic detergent, oleyl-polyoxyethylene glycol-ether. Because of the more widespread use of detergents in the pharmaceutical industry, such investigations are of integral importance. In addition to the usual data of toxicity studies, investigations of the effect of the compound on the blood pressure and the tissue aldehydes of the animals were conducted.

IN THE COURSE of a search for a suitable nonionic detergent to be used in pharmaceutical preparations, oleyl-polyoxyethylene glycol-ether (referred to as OPGE) suggested itself because of its appropriate physicochemical properties. The chemical formula of the compound is $C_{18}H_{35}OCH_2CH_2O(CH_2CH_2O)_{18}CH_2CH_2OH$. It is described by its manufacturer as a nonionic emulsifier and disperser composed of an oleic acid radical and polyoxyethylene glycol.[1] The compound is a white waxy paste, stable to acids and alkalis, and water soluble.

Before its contemplated use in pharmaceuticals, pertinent toxicity studies were indicated to rule out any potential animal toxicity for the compound even in dosages far in excess of the ones used eventually in practice. In our special case, the maximum daily intake would not exceed two milligrams.

The study of the toxicity of the compound was divided into a single dose phase, tested by intraperitoneal injections; and a repeated dose phase, tested by administering the compound contained in different concentrations in drinking water. A total of 196 Wistar strain rats was used, equally divided between male and female, for the single dose experiment, and 48 weanling animals for the repeated dose experiment.

EXPERIMENTAL

Single Dose Toxicity Study.—The following method was used for the determination of the LD_{50} of OPGE. Each essential concentration was tested on at least eight adult animals weighing between 140 and 350 Gm. The amount of solution injected intraperitoneally per animal varied with the individual weight; 1 cc./100 Gm. of body weight was administered. A preliminary survey to establish the dosage range was performed in groups of four, each time equally divided between males and females. It was found that the intraperitoneal injections *per se*, as injections of varied amounts of saline, had no fatal effect on the animals. It was further found that OPGE had a highly irritating effect when injected in concentrations of 2.5% in water either on muscle tissue or on the peritoneum. Necrosis and sloughing of the muscle and formation of fibrinous adhesions in the peritoneal cavity were the consequences. These effects were found especially in those animals not killed by the compound within forty-eight hours. The immediate symptoms of all the animals from the intraperitoneal injections were drowsiness, piloerection, watery nasal discharge, and loss of muscle control. Death occured with convulsions and opisthotonos, most likely due to respiratory depression. The animals were observed for forty-eight hours, if not dead within that time, they were sacrificed and autopsied. Main gross pathological findings were sanguinous fluid collected in the peritoneal cavity, and intestinal loops contracted and hemorrhagically infiltrated. No pleural effusion was present. In all surviving animals the livers showed areas of localized ischemia. Table I gives the dosage of OPGE injected and the percentage of dead animals of the total injected group. Since no difference was found in their reaction, both male and female animals are not separated in this report. The determination of the LD_{50} for OPGE was done by the method of Bliss (1) using probability units. The LD_{50} for OPGE on rats by intraperitoneal injection is 0.235 Gm./Kg. of body weight.

Repeated Dose Toxicity Study.—Forty-eight weanling rats weighing between 40 and 80 Gm., equally divided between male and female, were grouped as four experimental units of 12 each, and kept in an air-conditioned room at an average temperature of 72°. Six animals were housed in each cage until their individual weight reached 200 Gm.; thereafter three animals were caged together. Individual weights and the fluid intake per cage were recorded weekly. The average daily individual fluid consumption was derived by dividing the total amount of fluid intake per cage weekly by the number of inhabitants and the days of the week, assuming that each animal consumed approximately the same amount of fluid. After a period of four weeks of preliminary observation and the establishment of a base line for weight increase and fluid intake, the administration of OPGE was started in the following manner. The selection of the groups was completely arbitrary.

Group I —6 males, 6 females—receiving 0.1% OPGE dissolved in drinking water.

Group II — 6 males, 6 females—receiving 0.25% OPGE dissolved in drinking water.

Group III— 6 males, 6 females—receiving 0.5% OPGE dissolved in drinking water.

* Received December 13, 1952, from the Research Laboratories of McKesson & Robbins, Inc., Bridgeport, Conn.

[1] The compound will be abbreviated during the course of this report as OPGE, utilizing the initials of the chemical components for descriptive purposes.

This compound is manufactured by Antara Products, Inc., New York, N. Y.

TABLE I.—DETERMINATION OF LD_{50} OF OPGE

Dose, Gm./Kg.	No. Animals Used—Males	No. Animals Used—Females	Ratio Dead/Total—24 Hr.	Ratio Dead/Total—48 Hr.	Dead, %	Remarks
2.5	2	2	4/4	4/4	100	....
1.25	4	4	7/8	8/8	100	....
0.625	4	4	7/8	7/8	87.5	1 not i. p.[a]
0.3125	4	4	7/8	7/8	87.5	1 not i. p.
0.29	4	4	8/8	8/8	100	....
0.27	4	4	6/8	7/8	87.5	....
0.26	8	8	11/16	14/16	87.5	....
0.25	18	18	20/36	24/36	66.6	....
0.24	24	24	29/48	30/48	62.7	....
0.23	8	8	2/16	5/16	31.25	....
0.22	4	4	2/8	3/8	37.5	....
0.21	4	4	1/8	2/8	25	....
0.20	4	4	0/8	0/8	0	....
0.125	4	4	0/8	0/8	0	....
0.10	2	2	0/4	0/4	0	....

[a] Injection was intramuscular.

Group IV— 6 males, 6 females—receiving tap water (Controls).

The administration of OPGE in the drinking water was preferred to eliminate a possible factor of food refusal which might exist in any experimental procedure dealing with voluntary food selection, such as mixing the test substance with solid food. It was found to be much simpler and more accurate to control and measure the intake of fluid than that of food because of the unavoidable spillage of food by the animals. The diet remained unchanged throughout the experiment. The recording of weights and fluid intake was continued in the same manner as during the pretesting period. During the actual test period of six months, the blood picture and the blood pressure of the animals were studied. Blood counts consisted of the determination of the red cell count, the white cell count, the hemoglobin, and the differential count of the white cells. The blood was drawn from the nicked tip of the tail in the anesthetized animals. The systolic blood pressure was determined at monthly intervals by the plethysmographic method of Williams, Harrison and Grollman (2). The external appearance of the rats was essentially normal during the entire test period. The excreta remained of normal consistency. After six months, the animals were sacrificed and autopsied. The gross pathology was studied and frozen sections were made from the adrenal glands and kidneys. Sections of heart, lung, liver, spleen, adrenal, and kidney were preserved in 4% formaldehyde for paraffin sections. The hematoxylin-eosin stained slides were examined microscopically for eventual organ pathology.

Results.—Figure 1 shows the average weight gain of each group of 6 animals over a six-month period. There was a retardation of gain in the group receiving 0.5% OPGE even though the average weight of this group was slightly below that of the other groups at the beginning of the experiment. The groups of male animals administered 0.1% and 0.25% OPGE appear to have gained more weight than the controls; in the females, the weight curves were practically parallel throughout.

Figure 2 demonstrates the average fluid consumption per cage over the entire test period. It can be clearly seen that the control animals drank more than those on various concentrations of OPGE. It also can be seen that the higher concentration of OPGE in water decreased the total fluid intake considerably; and to such an extent that at one particular point, in one male group, the fluid consumption was less than 50% that of the control groups. The decreased fluid intake might have been due to the aversion of the animals to the soapy taste of the solutions.

From the average daily fluid intake, the average amount of OPGE taken in by each animal could be calculated. During the twenty-seven weeks of the test, each male rat of Group I averaged 22.04 cc. of fluid per day and each female of Group I averaged 21.08 cc. of fluid per day. Since each cubic centimeter contains 1 mg. of OPGE (0.1% solution), each male rat in this group consumed about 22.04 mg. of OPGE per day and each female animal, 21.08 mg. of the compound. The figures for the other groups as well as the total calculated intake of OPGE over the entire test period are given in Table II.

TABLE II.—CALCULATED FLUID AND OPGE INTAKE PER ANIMAL

Group	Sex	Average Fluid Intake Per Day, Cc.	Calculated Av. Daily Intake of OPGE, Gm.	Total OPGE Intake Over Test Period, Gm.	Calculated OPGE Intake Daily, Gm./Kg.
I	Female	21.08	0.02	22.97	0.02
	Male	22.04	0.02	24.32	0.02
II	Female	20.04	0.05	56.94	0.04
	Male	20.82	0.05	58.48	0.03
III	Female	15.33	0.07	83.42	0.07
	Male	16.34	0.08	91.61	0.07
IV	Female	31.68	Controls	...	...
	Male	24.72	Controls	...	...

The range of blood counts in all groups are given in Table III.

The average blood pressure readings for all four groups were as follows:

	Average Blood Pressure Reading
Group I	
Males	125.5 Hg.
Females	111.5 Hg.
Group II	
Males	126.8 Hg.
Females	107.3 Hg.
Group III	
Males	124.1 Hg.
Females	110.6 Hg.
Group IV	
Males	129.1 Hg.
Females	110.4 Hg.

There were no differences among the various groups. The variations and fluctuations were within the same range.

Autopsies were performed on all 47 surviving animals. After two months on test, one animal of the control group died of labyrinthitis. Macro-

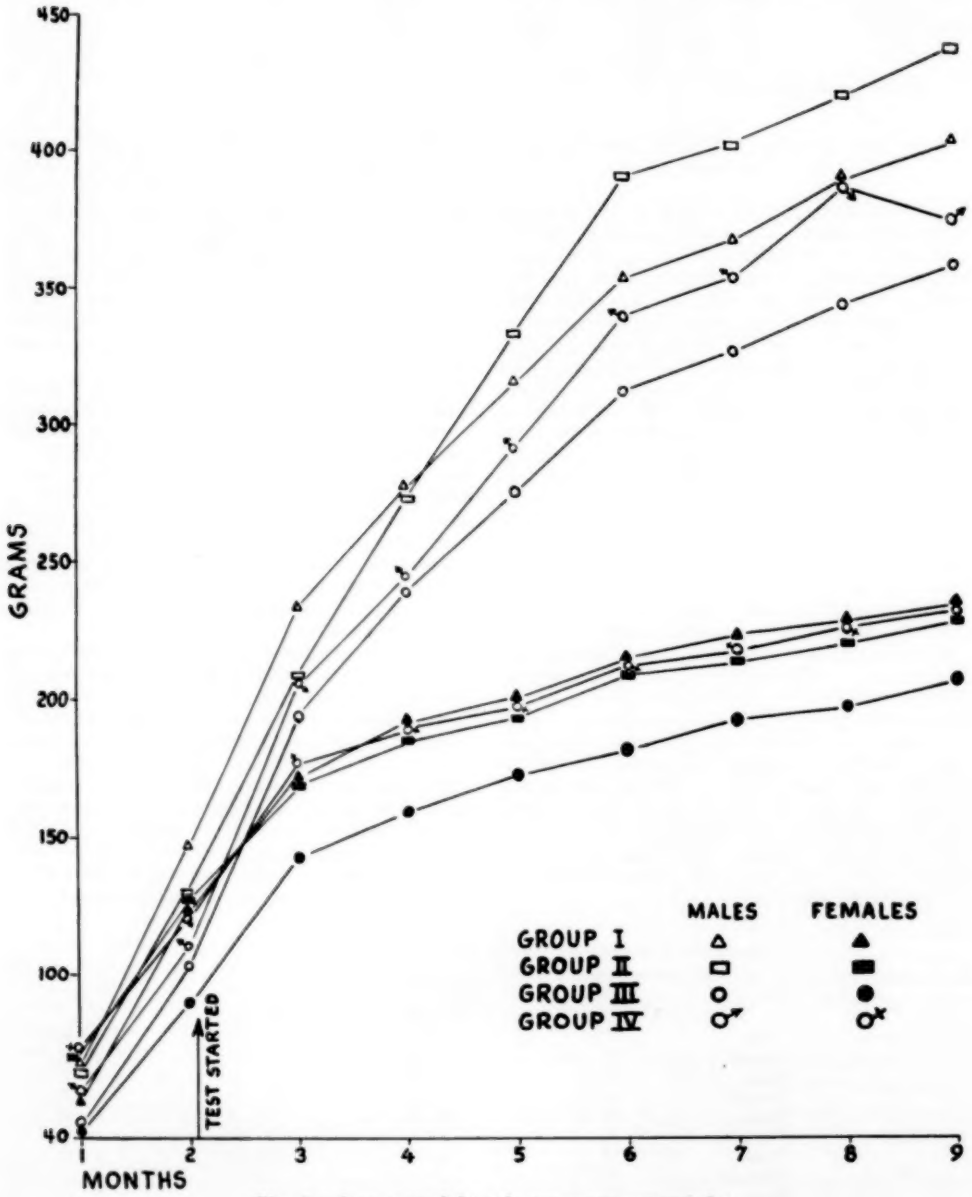

Fig. 1.—Average weight gain per group per month.

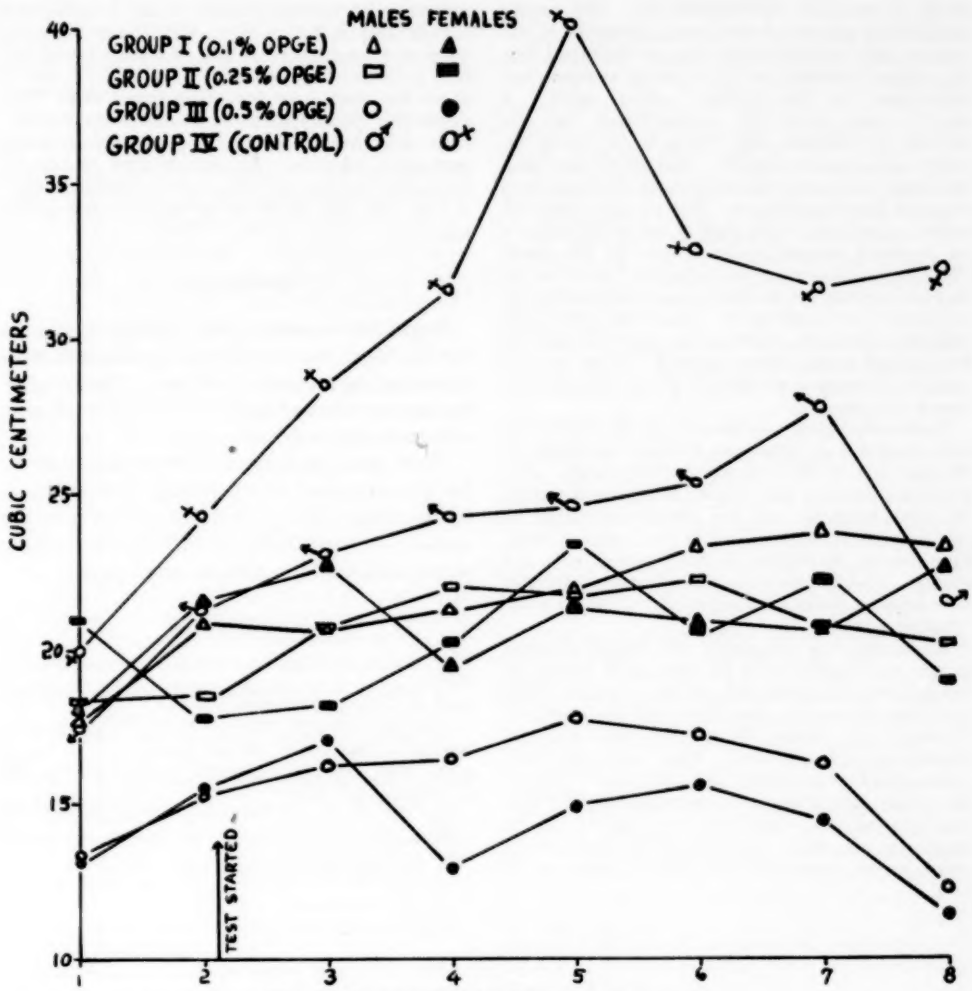

Fig. 2.—Average fluid intake per rat per month.

scopic examination revealed no difference in organ pathology between control and treated groups. One animal in the control group had splenomegaly; the reason for this could not be elicited.

The frozen sections of the adrenal gland and the kidney showed no difference in histochemical architecture as described by Oster (3). The shift of the tissue aldehydes in the intercorticomedullary zone in the female could very well be observed in correlation with the estrus cycle, a finding indicating that OPGE does not suppress the balance of the sex hormones in the test animals. The findings of Ehrlich and Waelsch (4) that surface-active agents suppress the stainability of aldehydes with fuchsin sulfurous acid stain could be confirmed in both the repeated dose and the single dose experiments, but more

TABLE III.—RANGE OF BLOOD COUNTS

Group	Red Cells (Thousands)	White Cells (Thousands)	Hemoglobin (Gm.)	Differential White Cells — Polymorphonuclear Cells				
				Neutrophils, %	Eosinophils, %	Basophils, %	Lymphocytes, %	Monocytes, %
Normal range[a]	7,000–10,000	8–19	15.6 Gm. 100%	2–25	0–4	0–1	55–96	0–3
I	9,400–11,600	8.9–14.9	15.38–17.0	14–22	0–1	0–4	67–77	4–10
II	9,350–11,200	9.2–13.5	14.82–17.63	8–22	0–1	0–4	68–85	3–9
III	8,400–11,106	10.7–15.7	13.71–16.51	14–24	0–2	0–3	68–74	4–9
IV Control	8,800–10,600	9.6–18.4	14.73–17.61	12–22	0–1	0–4	68–79	4–9

[a] See reference (5).

clearly in the single dose experiment. The normal distribution pattern of the tissue aldehydes in the various zones of the kidney was not disturbed, but the general intensity of the staining reaction was diminished by the surface active agent. A better stain could be accomplished by the method of Ehrlich and Waelsch (4) using an acetic acid prepared reagent. Essentially the tissue aldehydes were undisturbed in both the single and repeated dose experiments. The average blood cell values in this series were slightly out of the range of the reported normal values stated in the book, "The Rat in Laboratory Investigation," as far as the count of the monocytes and basophil polymorphonuclear cells were concerned. There was very little difference, however, between the controls and the test groups in all values reported. There was no apparent hemoconcentration in the groups with the lowest fluid intake.

Histopathological examination of the organ sections revealed no abnormal findings ascribable to the ingestion of OPGE in the drinking water. Few abnormal findings, like cellular infiltrations around the renal glomeruli and few descending tubuli, as well as small cell infiltrations in the liver and lungs, were evenly distributed in both control and test groups. No histopathological changes whatever were found in the group receiving 0.1% OPGE in the drinking water.

Discussion.—In comparing the results obtained in this investigation with the findings in studies by other authors of compounds of similar nature, there is a close agreement, especially with the study by Fitzhugh and Nelson (6) on polyethylene-glycol-monoisooctyl-phenol-ether. They state that in a sixteen-week experiment, this chemical decreases the growth-rate of rats in a concentration of 1% or more in the diet. Woodard and Calvery (7), in comparing the single dose toxicity of some surface active agents, report that the LD_{50} of sodium lauryl sulfate, by intraperitoneal route, is 0.21 Gm./Kg. and that of soap, 0.98 Gm./Kg. OPGE is in the same range of acute toxicity as that of sodium lauryl sulfate, a compound accepted in U. S. P. XIV, and is about four times more toxic than actual soap. The single dose toxicity seems to be due to the extreme tissue irritation caused by the compound on intraperitoneal injection. An unpublished report by Shelanski (8) gives an LD_{50} on oral administration, of 2.25 Gm./Kg. of OPGE on both rats and guinea pigs.

SUMMARY

Single and repeated dose toxicity studies on OPGE (oleyl-polyoxyethylene-glycol-ether) were performed on a total of 244 rats. The LD_{50} for the compound was found to be 0.235 Gm./Kg. on intraperitoneal injection.

There were no signs of repeated dose toxicity for the compound in six months when taken in concentrations of 0.1% and 0.25% in the drinking water. A concentration of 0.5% in the drinking water caused a slight retardation of growth.

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The Effects of Fasting Treatments on the Acid-Soluble Phosphate Fraction of the Small Intestine of the Rat*

By DONALD C. KROEGER† and LEROY D. EDWARDS

Studies were made in which rats were subjected to presacrificing fasting and subsequent feeding treatments in order to determine the effects of such treatments on the acid-soluble phosphate fraction of the combined mucosa and muscularis of the small intestine. Fasting for periods of twenty-four and forty-eight hours produced a significant progressive decrease in the concentration of the total energy-rich phosphate. Presacrificing feeding of ten- and fifteen-minute durations, following the fast of forty-eight hours, showed a restoration of the values toward normal.

THE USE of surviving smooth muscle tissue, mainly from the uterus or the intestines, has been the basis for the primary elucidation of drug action and the assay for biological activity of many compounds. In obtaining intestinal segments for isolated strip studies the general practice is to allow the intestine to become fairly well freed of its contents by subjecting the animal to a fasting period. This fasting not only serves to clear the intestine but also to secure intestinal

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strips from a more uniform group of animals. Picchioni (1) reported that intestinal strips showed greater activity if rats were fed just prior to sacrificing.

The present theories concerning the bioenergetics of muscular activity are based upon the findings that certain members of the organically bound phosphates have a relatively high free-energy change concerned with the hydrolysis of their phosphate linkages. Two of the most important compounds are creatine phosphate and adenosine triphosphate which are isolated from the acid-soluble phosphate fraction of the metabolic phosphates.

The object of this study was to determine by means of analysis of the acid-soluble phosphate distribution of intestinal strips whether there is any justification for a particular presacrificing treatment.

EXPERIMENTAL

The animals used in these studies were albino rats, Wistar Strain Purdue University, of both sexes ranging from 150 to 230 Gms. The rats were assembled and randomized with regard to weight and sex. The rats were sacrificed by cervical dislocation, immediately placed in a deep freeze and the neck severed to permit bleeding. The abdominal cavity was exposed, the small intestine removed, the lumen washed with ice-cold saline and the intestine cut into four equal lengths. The segments were trimmed of the mesenteric tissue, slit longitudinally, and the lumen blotted with absorbent toweling. The segments were then frozen with liquid nitrogen and powdered by the method of LePage (2). For studies on the musculature of the intestine, the mucosa was removed from the frozen strips by means of a razor blade (3) before powdering.

The powdered tissue was placed in a tared centrifuge tube containing ice-cold 10% trichloracetic acid and the sample weight determined by difference on a magnetic damped chainomatic balance. This suspension was extracted twice by centrifuging at 0° for fifteen minutes, the supernatant combined in a volumetric flask and diluted to volume with ice-cold 5% trichloracetic acid. The assays for the acid-soluble phosphate were made by the method of Ernster, *et al.* (4). In this study a Coleman Universal Spectrophotometer was used and transmission readings were made at 625 mμ.

This study consisted of four parts: (I) the determination of the acid-soluble phosphate fraction of the small intestine, divided into quarters, of rats fed *ad libitum;* (II) the determination of the acid-soluble phosphate fraction in terminal ileum strips of rats subjected to twenty-four and forty-eight hour fasting periods; (III) the determination of the acid-soluble phosphate fraction in terminal ileum strips of rats subjected to a forty-eight hour fast and fed for intervals of ten and fifteen minutes prior to sacrificing; and (IV) the determination of the acid-soluble phosphate fraction in the muscularis of terminal ileum strips of rats subjected to a forty-eight hour fast.

RESULTS

The abbreviations used in the subsequent text for the various acid-soluble phosphate compounds are: I. P.–P. for the orthophosphate phosphorus present in the tissues as such, Cr. P.–P. for the phosphate hydrolyzed at room temperature in 1 *N* sulfuric acid for thirty minutes which is creatine phosphate phosphorus, A. T. P.–P. for the phosphate hydrolyzed at 100° for twenty minutes in 1 *N* sulfuric acid which is composed of two-thirds of the phosphate of adenosine triphosphate and one-half of the phosphate from adenosine diphosphate, ~ P.–P. for energy-rich phosphate phosphorus which is composed of the sum of the values obtained for Cr. P.–P. and A. T. P.–P., T. A. S. P.–P. for the total acid-soluble phosphate phosphorus extracted by trichloracetic acid which is composed of I. P.–P., Cr. P.–P., A. T. P.–P. and other phosphate compounds resistant to hydrolysis at 100° for twenty minutes in 1 *N* sulfuric acid.

In order to characterize the phosphate hydrolyzed under the various conditions referred to above, hydrolysis curves were prepared for the phosphate hydrolyzed at room temperature, 25° in 1 *N* sulfuric acid for thirty minutes and for the phosphate hydrolyzed at 100° for twenty minutes in 1 *N* sulfuric acid. Hydrolysis curves for Cr. P.–P. and A. T. P.–P. are shown in Figs. 1 and 2.

The concentration means of the various acid-soluble phosphate compounds in the different sections of the intestines of rats as influenced by feeding and fasting conditions are shown in Table I. Analysis of variance was applied to the values obtained for Intestinal Sections I, II, III, and IV in fed *ad libitum* treatment and a summary of the statistical data is shown in Table II. Only inorganic phosphate and creatine phosphate show significant changes; hence, for rats fed *ad libitum* prior to sacrificing, there appears to be significantly greater amounts of creatine phosphate in the oral end of the small intestine than in the aboral end. Although significance was obtained for the various sections with respect to inorganic phosphate, the pattern is less pronounced than in the case of creatine phosphate.

Table I and Table II also contain the concentration means and the summary of the analyses of variance and significant differences for the various acid-soluble phosphates determined for the terminal ileum strips of rats fed *ad libitum*, fasted for twenty-four hours, fasted for forty-eight hours, fasted for forty-eight hours and fed ten and fifteen minutes prior to sacrificing.

The graph in Fig. 3, which shows the relationship of the amounts of ~ P.–P., A. T. P.–P. and Cr. P.–P. with respect to the time factor of the various treatments, indicates that as the time of fasting lengthens the amounts of Cr. P.–P. decrease and the A. T. P.–P. increase. The fasting treatment causes a reversal in the ratio of these two compounds and the brief feeding interval tends to restore the ratio to that found in *ad libitum* fed rats. The pattern for ~ P.–P. shows a decrease of these compounds with increased duration of fasting and a shift toward *ad libitum* feeding values with the brief feeding treatments. When assays were made on the forty-eight hour fast plus ten and fifteen minute feeding treatment, note was made of the distance that the freshly eaten food had traveled in the

TABLE I.—CONCENTRATION MEANS OF THE VARIOUS ACID-SOLUBLE PHOSPHATE COMPOUNDS IN THE DIFFERENT SECTIONS OF THE INTESTINES OF RATS AS INFLUENCED BY FEEDING AND FASTING CONDITIONS[a]

Study Part[b]	Number of Rats	Intestinal Sections[c]	Mg. % Phosphorus (Wet Weight of Tissue)				
			T. A. S. P.-P.	I. P.-P.	Cr. P.-P.	A. T. P.-P.	~ P.-P.
A_1	10	I	100.24 ± 4.46	24.86 ± 1.04	13.06 ± 0.62	6.86 ± 0.62	19.92 ± 1.32
..	..	II	102.46 ± 4.46	23.76 ± 1.04	9.90 ± 0.62	8.21 ± 0.62	18.11 ± 1.32
..	..	III	98.68 ± 4.46	22.02 ± 1.04	11.18 ± 0.62	7.28 ± 0.62	18.46 ± 1.32
..	..	IV	103.08 ± 4.46	22.86 ± 1.04	10.18 ± 1.76	7.01 ± 1.10	17.18 ± 2.02
B_1	10	IV	104.88 ± 4.72	26.38 ± 1.01	6.05 ± 0.50	9.63 ± 0.63	15.68 ± 1.01
B_2	10	IV	101.37 ± 3.67	25.74 ± 0.90	3.23 ± 0.33	10.68 ± 1.01	14.18 ± 1.04
C_1	10	IV	116.19 ± 6.76	25.53 ± 1.08	6.01 ± 0.23	9.98 ± 1.22	15.99 ± 1.62
C_2	10	IV	100.50 ± 4.09	24.04 ± 0.97	5.75 ± 0.98	10.69 ± 0.70	16.44 ± 0.82
D_1	10	IV	135.56 ± 6.67	27.27 ± 1.64	10.01 ± 1.22	7.48 ± 1.51	17.59 ± 2.23

[a] *S. E. M.* = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

[b] A_1 = *Ad libitum* fed, B_1 = 24 hour fast, B_2 = 48 hour fast, C_1 = 48 hour fast—10 min. feeding, C_2 = 48 hour fast—15 min. feeding, D_1 = 48 hour fast, muscularis.

[c] Intestinal Sections: I = First Quarter (oral), II = Second Quarter, III = Third Quarter, IV = Fourth Quarter (aboral).

gastro-intestinal tract. In no case was food found farther than the first 15 cm. of the small intestine and in a number of cases the duodenum was still devoid of food. Thus some mechanism appears to be present which causes the terminal ileum strips to change with regard to the high energy phosphates when food is present in the stomach or the upper intestinal region.

The fasting treatments tend to decrease the variance of the means of Cr. P.-P. and A. T. P.-P. and with regard to these compounds the forty-eight hour fast group of animals was more uniform than those receiving other treatments.

The analysis of variance made on the means of the concentrations of the acid-soluble phosphate compounds obtained from the whole intestine mucosa and muscularis, and the intestinal musculature of animals fasted for forty-eight hours showed that the muscularis contained significantly relative larger amounts of T. A. S. P.-P. than the mucosa and muscularis combined. The amounts of Cr. P.-P. in the muscularis were relatively greater in the same respects. The other compounds failed to show significance for the treatments. Thus it would appear from these results that the mucosa at the forty-eight hour fast period was causing a dilution effect upon the amounts of Cr. P.-P. and T. A. S. P.-P. present in the intestinal musculature.

DISCUSSION

The values for the total acid-soluble phosphate are composed of the values of inorganic phosphate, creatine phosphate, adenosine triphosphate, and other phosphate compounds whose linkages are resistant to acid-hydrolysis used to determine the phosphate hydrolyzed at 100° for twenty minutes. The difference between the total acid-soluble phosphate and the phosphate hydrolyzed at 100° for twenty minutes represents the phosphate of a

TABLE II.—SUMMARY OF THE STATISTICAL EVALUATION OF THE DATA FOR THE VARIOUS ACID-SOLUBLE PHOSPHATE COMPOUNDS IN THE DIFFERENT SECTIONS OF THE INTESTINES OF RATS AS INFLUENCED BY FEEDING AND FASTING CONDITIONS[a]

Intestinal Section or Study Part	Statistical Treatment	Acid-Soluble Phosphates				
		T. A. S. P.-P.	I. P.-P.	Cr. P.-P.	A. T. P.-P.	~ P.-P.
Sections of A_1		*[b]	4.14[c]	5.70[d]	*	*
I *vs.* II, III & IV	Analysis of Variance (*F*-ratio)	*	5.55[c]	*	*	*
IV *vs.* II & III		*	*	*	*	*
II *vs.* III		1.74	12.73[d]	*	*	*
I *vs.* II		*	*	3.54[d]	*	*
I *vs.* III	Significant Difference	*	*	2.00	*	*
I *vs.* IV		*	*	3.24[d]	*	*
II *vs.* III	(Fisher's *t*)	*	*	1.44	*	*
II *vs.* IV		*	*	*	*	*
III *vs.* IV		*	*	1.12	*	*
Treatments	Analysis of Variance (*F*-ratio)	1.54	1.63	3.35	2.85	*
A_1 *vs.* B_1		*	2.22[c]	*	2.36[c]	*
A_1 *vs.* B_2	Significant Difference	*	*	3.59[d]	*	*
A_1 *vs.* C_1		*	*	*	2.28[c]	*
A_1 *vs.* C_2	(Fisher's *t*)	*	*	2.05[c]	2.92[c]	*

[a] For explanation of intestinal sections, study parts, and feeding or fasting treatments see Table I.

[b] * indicates that the values are less than 1.0.

[c] Indicates significance at the 0.05 level.

[d] Indicates significance at the 0.01 level.

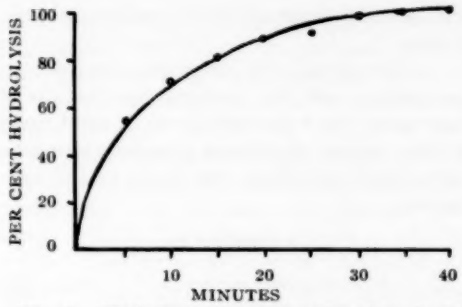

Fig. 1.—Hydrolysis of creatine phosphate in 3.0 ml. 5% trichloracetic acid aliquot of rat ileum with 0.5 ml. 10 *N* sulfuric acid at 20° C.

group of compounds, mainly phosphorylated ribose sugars, possessing the normal ester type phosphate linkage.

The values obtained for A. T. P.–P. represent two-thirds of the phosphate of adenosine triphosphate and one-half of the phosphate of adenosine diphosphate, the residual phosphate of these two compounds being with difficulty hydrolyzed by acid. However, some of the value for A. T. P.–P. may be due to the hydrolysis of small amounts of hexosediphosphate, glucose-1-phosphate, and other labile phosphate esters. Rowles and Stocken (5) pointed out that in the analysis of adenosine triphosphate the hydrolysis should be brought about by the specific enzyme as well as acid hydrolysis for best results. The hydrolysis of creatine phosphate in acid molybdate should not proceed beyond the thirty-minute time interval as suggested by Ernster, *et al.* (4), since A. T. P. and A. D. P. begin to hydrolyze at room temperature beyond this time interval (6).

The removal of the mucosa from the muscularis was perhaps the greatest source of error in this study since this removal without causing trauma to the muscle is extremely difficult. Furchgott (7) reported a method for removing the mucosa from rabbit intestinal strips but this method could not be adapted for use on rat intestine. The method used by Walaas and Walaas (3) for the removal of endometrium from uterine smooth muscle was found most adaptable for rat intestinal strips and was therefore used in these studies.

Due to the extreme sensitivity of the acid-soluble phosphate compounds to enzymatic and chemical decomposition, it is necessary that all steps in the preparation and assay of intestinal strips be performed at low temperatures, about 0°. LePage (2) suggested that the animal be slightly anesthetized with Nembutal and then quickly frozen by immersing in liquid air or nitrogen until the internal organs are frozen. Such a procedure is not applicable for use with intestinal studies since this tissue contains fecal material which makes it necessary to remove the intestines and cleanse the lumen before freezing is attempted. This necessitated delay is also a source of error.

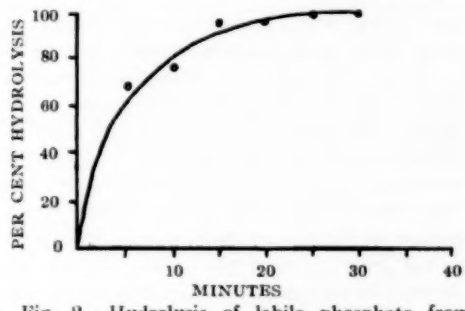

Fig. 2.—Hydrolysis of labile phosphate from A. T. P. and A. D. P. in 3.0 ml. 5% trichloracetic acid aliquot of rat ileum with 0.5 ml. 10 *N* sulfuric acid at 100° C.

Lundsgaard (8) studied the acid-soluble phosphate fraction of the intestinal mucosa of rats fasted for twenty-four hours and found that the concentration of T. A. S. P.–P. averaged 459 mg. % phosphorus per dry weight of tissue. This amount is approximately 80 to 90 mg. % phosphorus per wet weight of tissue which corresponds to the values found in this study. Lundsgaard also reported that the absorption of glucose by the intestinal mucosa caused a decrease in the amounts of inorganic phosphate and an increase in the amounts of the labile phosphates. He believed these changes were local effects shown only by those areas of the mucosa involved with the absorption of food. In this study newly ingested food had not yet arrived at the section of the intestines that were analyzed. The distance between the newly ingested food in the upper intestines and the strips taken for assay was 30 to 40 cm.

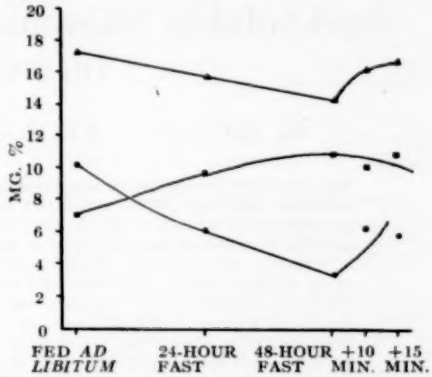

Fig. 3.—Concentration means, mg. %, of the energy-rich phosphate compounds in terminal ileum strips of rats *vs.* presacrificing feeding or fasting treatments.

Upper curve: ~P.–P.; middle curve: A.T.P.–P.; bottom curve (second from top starting at the left): Cr.P.–P. + 10 and + 15 = post fast feeding.

The presence of food in the stomach or the upper intestinal tract causing a change in the phosphate values of the terminal ileum strip suggests some mechanism is involved in these changes. Whether this is of a nervous or hormonal nature is impossible to state. Grossman (9) discussed the question of a hormone which effects intestinal motility and concluded that the evidence to date does not establish that there is such a hormone but that the possibility does exist. This phenomenon is perhaps similar to the mechanism which causes the mass movements of the large intestine upon the entrance of food into the stomach.

SUMMARY

1. The study of the four quarters of the small intestine, mucosa, and muscularis, of rats showed that the oral end had significantly greater amounts of creatine phosphate than the aboral.

2. Increased periods of fasting up to forty-eight hours caused a significant reduction in the amounts of creatine phosphate and energy-rich phosphate. These same fasting treatments caused a significant increase in the amounts of adenosine triphosphate.

3. The variance for the concentration means of creatine phosphate and adenosine triphosphate were reduced by the twenty-four hour and the forty-eight hour fasting periods.

4. Feeding rats, fasted for forty-eight hours, for periods of ten and fifteen minutes caused a significant change in the amounts of creatine phosphate and adenosine triphosphate present in the terminal ileum segments, mucosa and muscularis.

5. Comparing the intestine, mucosa and muscularis, with the intestinal musculature of rats fasted for forty-eight hours a significantly greater amount of creatine phosphate and total acid-soluble phosphate was found in the musculature.

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The Effects of Some Metabolites and Anoxia on the Acid-Soluble Phosphate Fraction of *in Vitro* Rat Intestinal Strips*

By DONALD C. KROEGER,† and LEROY D. EDWARDS

Rat intestinal strips were suspended in a muscle bath containing Kreb-Henseleit solution and then were subjected to either endogenous substrate depletion and metabolite restoration or to periods of anoxia and oxygen restoration. Acid-soluble phosphate determinations were performed upon the intestinal muscularis at various times during their incubation. The intestinal strips were suspended upon holders which approximated the typical laboratory setup for the recording of the longitudinal muscle contractions. Substrate depletion for a period of one hour indicated a progressive decrease in the energy-rich phosphates and an increase in the inorganic phosphate. Anoxic conditions were studied, using Kreb-Henseleit solution containing glucose, 100 mg. per cent, with and without thyroxin, 10^{-6} M. Solutions containing thyroxin appear capable of maintaining the intestinal strips more effectively.

RECENTLY THERE has been given increasing attention to a method for the study of the substrate requirements of *in vitro* smooth muscle tissue. This method was first reported in 1912 by Rona and Neukirch (1–3) and consisted of placing intestinal strips from rabbits in Tyrode's solution without glucose. There was considerable reduction of the amplitude of contractions of the longitudinal muscle in approximately one hour, during which time the tissue had been depleted of its endogenous substrate. When a 60 to 80 per cent reduction of amplitude was obtained, glucose was added and within a few minutes the spontaneous contractions were restored towards their original height. Using this technique numerous compounds have been tested by various investigators (4–7). Furchgott, *et al.* (6, 8), and West, *et al.* (7), have reported quantitative studies using various substrates with this method. The method has also been used for the determination of effects of metabolic inhibitors upon muscular activity (9–13), however, only the studies reported by

* Received August 22, 1952, from the Research Laboratories, Purdue University, School of Pharmacy, Lafayette, Indiana.
Presented to the Scientific Section, A. PH. A., Philadelphia Meeting, August, 1952.
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Salerno, *et al.* (14), and Furchgott and Shorr (15) mention any correlation of the intestinal activity with quantitative data concerning the levels of the various metabolic phosphate compounds. A similar technique utilized by Furchgott and Shorr (15) involved subjecting intestinal tissue to a period of anaerobiosis followed by oxygen restoration to study the effects on muscular activity.

The present study involved the analysis of the acid-soluble phosphate distribution of intestinal strips to determine the importance of the presence of metabolites and the importance of supplying oxygen to intestinal strips of rats suspended in a smooth muscle bath for periods of time ordinarily required for pharmacological tests.

EXPERIMENTAL

The animals used in these studies were albino rats, Wistar Strain Purdue University, of both sexes ranging from 150 to 230 Gm. The rats were assembled and randomized with regard to weight and sex. The rats were sacrificed by cervical dislocation and the intestines were exposed by laparotomy. The desired length of the terminal ileum, 25 to 30 cm., was removed and the lumen washed free of fecal material using warm physiological saline, 37°. After washing, the intestine was allowed to contract to its minimal length by placing in a beaker of warm saline, and then was divided into the desired strips. The strips were mounted on small wire holders which consisted of a 14-cm. long strip of wire having a loop handle at the top end and a hook at the other end. Ten centimeters from the hook a loop was formed in the wire to hold a thin rubber strip, surgical dam 2 mm. wide and 30 mm. long, with a small wire hook at the opposite end. The rubber strip was slightly stretched when a 5-cm. intestinal strip was mounted on the holder, thus allowing the smooth muscle to contract against tension. The holder containing the intestinal strip was placed in a muscle chamber similar to that described by Furchgott (12). Six muscle chambers of 25 ml. volume were placed in a constant temperature bath maintained at 37 ± 0.1°. The aeration gases used were either 95% oxygen-5% carbon dioxide or 95% nitrogen-5% carbon dioxide. By use of a circular manifold with connections to the hypodermic needle of each muscle chamber it was possible to maintain a uniform bubbling rate of eight to ten bubbles per minute per each chamber.

The bathing fluid was Kreb-Henseleit bicarbonate solution (16) to which the substrates glucose, sodium pyruvate, or anhydrous sodium acetate were added in final concentrations of 100 mg. %. Pyruvic acid was prepared by the method of Howard and Fraser (17) and the sodium salt by the method of Robertson (18).

At the specified assay time, the intestinal strip was removed from the muscle bath by means of the wire holder and immediately plunged into a beaker containing liquid nitrogen. While still frozen, the mesenteric fringes were removed, the tissue cut longitudinally along the line of mesenteric attachment and flattened on absorbent toweling with the serosa side down and the mucosa removed by means of a razor blade. The tissue was then powdered, extracted for acid-soluble phosphate and assayed as previously described (19).

Experimental Design of Study I.—The determination of the acid-soluble phosphate fraction of terminal ileum segments of rat intestine subjected to endogenous substrate depletion and exogenous substrate restoration consisted of three parts: (*a*) restoration by glucose; (*b*) restoration by sodium acetate; and (*c*) restoration by sodium pyruvate. Table I describes the treatments to which the ileal strips of rat intestine were subjected.

TABLE I.—DESCRIPTION OF TREATMENTS TO WHICH ILEAL STRIPS OF RAT INTESTINE WERE SUBJECTED FOR STUDY I

Treatment Number	Description
1	Strip placed in muscle chamber containing glucose 100 mg. % for thirty minutes and then assayed
2	Strip placed in muscle chamber containing no substrate for ninety minutes, then assayed
3	Strip placed in muscle chamber containing no substrate for ninety minutes, glucose 100 mg. % added and then assayed at one hundred and twenty minutes
4	Strip placed in muscle chamber containing glucose 100 mg. % and assayed at one hundred and twenty minutes
5	Strip placed in muscle chamber containing no substrate for ninety minutes, sodium acetate 100 mg. % added and assayed at one hundred and twenty minutes
6	Strip placed in muscle chamber containing sodium acetate 100 mg. % and assayed at one hundred and twenty minutes
7	Strip placed in muscle chamber containing no substrate for ninety minutes, sodium pyruvate 100 mg. % added and assayed at one hundred and twenty minutes
8	Strip placed in muscle chamber containing sodium pyruvate 100 mg. % and assayed at one hundred and twenty minutes

Experimental Design of Study II.—The determination of the acid-soluble phosphate fraction of rat intestine when subjected to initial anaerobiosis and subsequent aerobiosis consisted of two parts: (*a*) the use of duodenal-jejunum strips maintained in either plain or thyroxin containing Kreb-Henseleit bicarbonate solution, and (*b*) the use of terminal ileum strips maintained in either plain or thyroxin containing Kreb-Henseleit bicarbonate solution. The physical set-up for this study was the same as previously described except an arrangement was present to permit the change of the aeration gases by means of a three-way stopcock; either 95% oxygen-5% carbon dioxide or 95% nitrogen-5% carbon dioxide mixtures could be readily admitted to the muscle chambers. Kreb-Henseleit bathing fluid with glucose, 100 mg. %, was used as the bathing medium. In some determinations, thyroxin, 10^{-6} molar in final concentration was added to the bath-

ing medium. Table II describes the treatments to which the duodenal-jejunum or the terminal ileum strips were subjected.

TABLE II.—DESCRIPTION OF TREATMENTS TO WHICH INTESTINAL STRIPS OF THE RAT WERE SUBJECTED FOR STUDY II

Treatment Number	Description
1	Strip placed in muscle chamber aerated with O_2-CO_2 for thirty minutes, then assayed
2	Strip placed in muscle chamber aerated with O_2-CO_2 for thirty minutes, then N_2-CO_2 for sixty minutes and then assayed
3	Strip placed in muscle chamber containing thyroxin and aerated with O_2-CO_2 for thirty minutes, then N_2-CO_2 for sixty minutes, and assayed
4	Strip placed in muscle chamber aerated with O_2-CO_2 for thirty minutes, then N_2-CO_2 for sixty minutes, then O_2-CO_2 restored for thirty minutes and assayed
5	Strip placed in muscle chamber aerated with O_2-CO_2 for one hundred and twenty minutes and assayed
6	Strip placed in muscle chamber containing thyroxin and aerated with O_2-CO_2 for thirty minutes, then N_2-CO_2 for sixty minutes, O_2-CO_2 restored for thirty minutes and assayed
7	Strip placed in muscle chamber containing thyroxin and aerated with O_2-CO_2 for one hundred and twenty minutes, then assayed

RESULTS

Study I.—The primary effect of added substrates to endogenous substrate depleted terminal ileum strips of rat intestine was one of increased tonus level followed by increased amplitude of contractions. This is shown in the photograph of a kymographic tracing in Fig. 1. The time required to obtain 70 to 80% reduction in contraction amplitude for rat intestine was eighty to ninety minutes, and the ninety-minute period was used as the depletion period for the phosphate assays in this study. Tables III and IV show the concentration means and the significant difference, respectively, for the various acid-soluble phosphate compounds of intestinal strips subjected to the treatments listed in Table I.

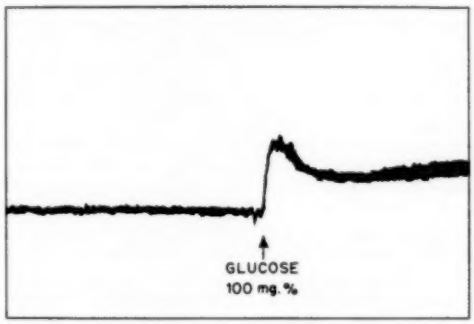

Fig. 1.—Photograph of a kymograph tracing showing the effects of exogenous glucose restoration after endogenous substrate depletion.

TABLE III.—CONCENTRATION MEANS OF THE VARIOUS ACID-SOLUBLE PHOSPHATE COMPOUNDS DETERMINED IN THE ILEUM STRIPS OF RAT INTESTINES SUBJECTED TO ENDOGENOUS SUBSTRATE DEPLETION AND EXOGENOUS SUBSTRATE RESTORATION[a,b]

Treatment Number	Mg. % Phosphorus (Wet Weight of Tissue)				
	T. A. S. P.–P.	I. P.–P.	Cr. P.–P.	A. T. P.–P.	~ P.–P.
1	104.73 ± 3.90	27.44 ± 1.02	4.85 ± 0.23	7.68 ± 0.53	12.63 ± 1.02
2	106.57 ± 8.37	30.41 ± 2.38	3.90 ± 0.56	5.27 ± 0.66	9.18 ± 0.61
3	97.75 ± 4.26	30.40 ± 1.27	3.99 ± 0.93	5.17 ± 0.71	9.15 ± 1.44
4	112.95 ± 2.38	31.66 ± 4.03	8.04 ± 2.53	6.27 ± 0.86	14.31 ± 3.38
5	93.88 ± 9.07	25.19 ± 3.07	5.98 ± 1.19	3.79 ± 0.53	9.77 ± 1.72
6	93.42 ± 6.70	26.19 ± 1.82	4.61 ± 1.52	4.82 ± 0.84	9.42 ± 2.32
7	94.42 ± 2.70	29.69 ± 2.99	5.15 ± 0.35	5.00 ± 1.21	10.15 ± 1.02
8	97.50 ± 6.24	26.68 ± 2.66	4.62 ± 0.34	4.71 ± 0.49	9.32 ± 0.65

[a] Treatment number corresponds to those in Table I.

[b] $S.E.M. = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

TABLE IV.—SIGNIFICANT DIFFERENCE, *t* TEST, FOR THE VARIOUS ACID-SOLUBLE PHOSPHATE COMPOUNDS IN THE ILEUM STRIPS OF THE RAT INTESTINE SUBJECTED TO ENDOGENOUS SUBSTRATE DEPLETION AND EXOGENOUS SUBSTRATE RESTORATION

Intestinal Strip	Degrees of Freedom	Acid-Soluble Phosphates				
		T. A. S. P.–P.	I. P.–P.	Cr. P.–P.	A. T. P.–P.	~ P.–P.
1 vs. 2	5	*[a]	*	*	1.13	1.44
3–4 vs. 5–6	3	*	1.08	1.20	1.72	1.34
3–4 vs. 7–8	3	*	*	*	*	*
5–6 vs. 7–8	3	*	1.34	1.72	4.21[b]	1.36

[a] *Indicates values less than 1.0.

[b] Indicates significance at the 0.05 level.

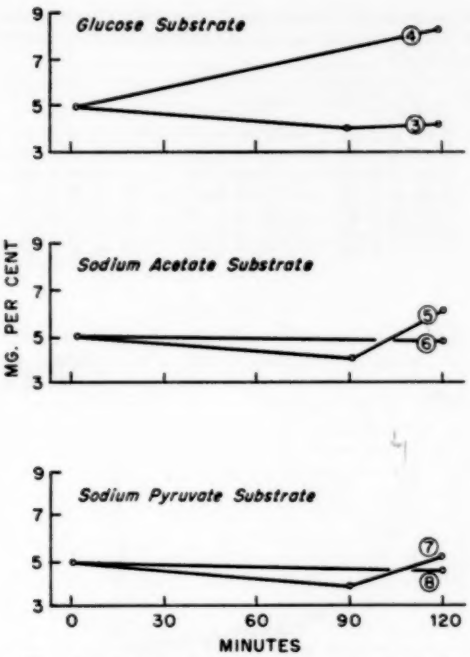

Fig. 2.—Concentration means, mg. %, of Cr. P. — P. in rat ileum strips subjected to endogenous substrate depletion and exogenous substrate restoration vs. time of incubation.

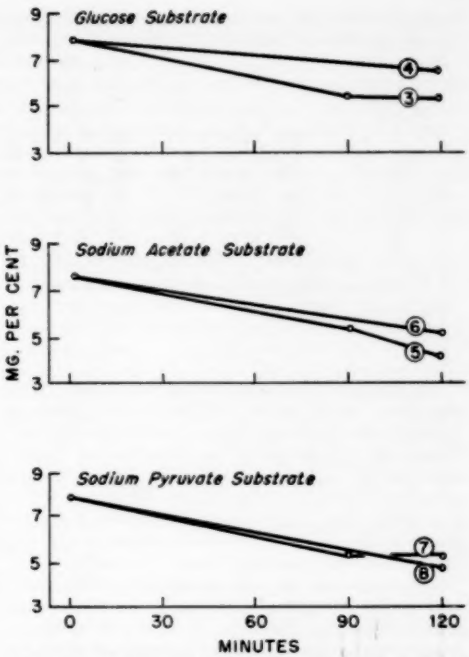

Fig. 3.—Concentration means, mg. %, A. T. P. — P. in rat ileum strips subjected to endogenous substrate depletion and exogenous substrate restoration vs. time of incubation.

Significant difference tests, *t* tests, were made on the means of these various phosphate compounds of strip 1 *versus* strip 2 and no significance was obtained. Tests for the remainder of the strips were not significant except A. T. P.- P. in strips 5 plus 6 *versus* 7 plus 8. The concentration means shown in Table III show a trend which is further illustrated by Figs. 2, 3, and 4. The values for the energy-rich phosphates decreased during the depletion period and the addition of a substrate tended to effect restoration. With the exception of glucose, the strips which had access to substrates throughout the two-hour period appeared to lose energy-rich phosphate. An indication was given that glucose tends to support the values for the energy-rich phosphates better than sodium acetate and sodium pyruvate over a two-hour period in a muscle bath.

Study II.—A photograph of a kymographic tracing of rat ileum subjected to anoxic conditions and subsequent restoration with oxygen is shown in Fig. 5. The tissue strip was allowed to contract in Kreb-Henseleit solution with glucose, 100 mg. %, aerated with 95% oxygen-5% carbon dioxide mixture for a period of thirty minutes, at which time this gas mixture was discontinued and 95% nitrogen-5% carbon dioxide was bubbled into the bath. The tonus drop was immediately evidenced but the strip still contracted during the anoxic phase. The intestinal strip was allowed to remain under anaerobic conditions for a period of one hour, after which time the nitrogen-carbon dioxide mixture was replaced by the oxygen-carbon dioxide mixture. The latter caused an immediate restoration of the tonus level.

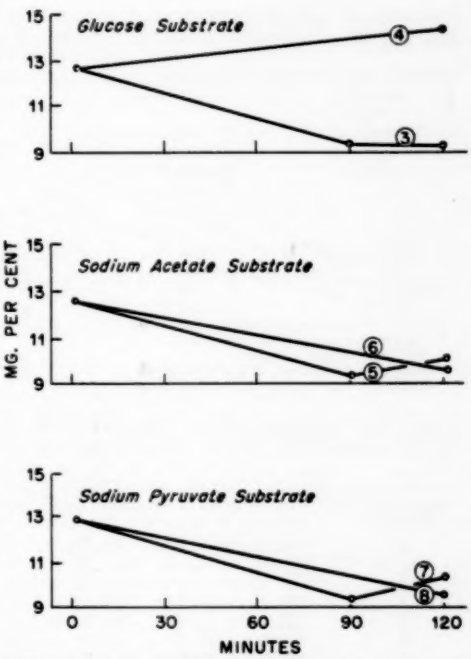

Fig. 4.—Concentration means, mg. %, ~ P. — P. in rat ileum strips subjected to endogenous substrate depletion and exogenous substrate restoration vs. time of incubation.

TABLE V.—CONCENTRATION MEANS OF THE VARIOUS ACID-SOLUBLE PHOSPHATE COMPOUNDS DETERMINED IN THE DUODENAL-JEJUNUM STRIPS OF RAT INTESTINES SUBJECTED TO INITIAL ANAEROBIOSIS AND SUBSEQUENT AEROBIOSIS[a,b]

Treatment Number	Mg. % Phosphorus (Wet Weight of Tissue)				
	T. A. S. P.–P.	I. P.–P.	Cr. P.–P.	A. T. P.–P.	~ P.–P.
1	121.12 ± 2.02	30.47 ± 1.23	7.96 ± 1.91	6.15 ± 0.70	14.11 ± 2.06
2	115.17 ± 3.07	32.47 ± 1.82	7.47 ± 1.49	4.18 ± 0.56	11.66 ± 1.56
3	120.52 ± 2.56	31.37 ± 0.99	9.84 ± 0.93	3.39 ± 0.97	13.43 ± 0.84
4	120.20 ± 3.91	32.38 ± 0.84	7.45 ± 0.47	4.51 ± 0.42	11.96 ± 0.80
5	119.97 ± 0.16	32.03 ± 1.19	8.90 ± 2.41	4.63 ± 0.51	13.53 ± 2.24
6	115.23 ± 4.72	27.18 ± 1.91	12.85 ± 1.49	4.71 ± 0.70	17.56 ± 1.02
7	120.97 ± 7.51	29.86 ± 0.94	11.41 ± 1.92	4.74 ± 1.11	16.48 ± 0.87

[a] Treatment number corresponds to those in Table II.

[b] $S.E.M. = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

The means of the concentrations of the various phosphate compounds obtained for duodenal-jejunum rat intestinal strips are shown in Table V. These data were analyzed by the method of Cochran and Cox (20). Table VI is a summary of the analyses of variance and significant differences for the treatment data. The almost complete lack of significance is due to a considerable extent to the large variance of the individual treatment means.

The means of the concentrations of the various acid-soluble phosphate compounds for the terminal ileum strips of rat intestine are shown in Table VII. A summary of the statistical analyses of these data is also shown in Table VI. Only the values for T. A. S. P.–P. and Cr. P.–P. are significantly influenced by the treatments, indicating that the amounts of T. A. S. P.–P. and Cr. P.–P. present in the strips undergoing no anoxia are greater than the amounts

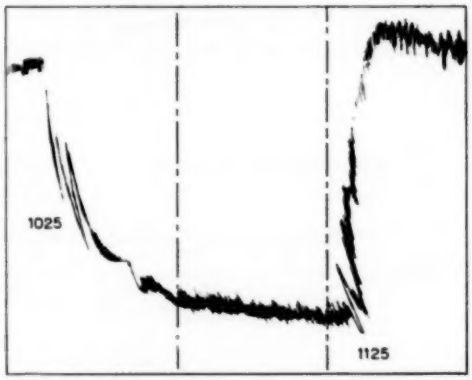

Fig. 5.—Photograph of a kymograph tracing illustrating the effects of subjecting rat ileum strips to a period of anoxia followed by oxygen restoration.

TABLE VI.—ANALYSIS OF VARIANCE, *F* RATIO, AND SIGNIFICANT DIFFERENCE, *t* TEST, FOR THE VARIOUS ACID-SOLUBLE PHOSPHATE COMPOUNDS IN RAT INTESTINAL STRIPS SUBJECTED TO AEROBIOSIS OR ANAEROBIOSIS AND SUBSEQUENT AEROBIOSIS

Intestinal Strips	Statistical Study	Acid-Soluble Phosphates				
		T. A. S. P.–P.	I. P.–P.	Cr. P.–P.	A. T. P.–P.	~ P.–P.
Duodenal–jejunum strips	Treatments (*F* ratio)	*[a]	1.52	1.14	*	3.96
	Strips (*t* tests)			not significant		
Ileum strips[b]	Treatments (*F* ratio)	20.11[d]	1.10	32.36[d]	*	1.28
	Strips (*t* tests)					
	4 *vs.* 5	*	*	5.17[c]	*	*
	4 *vs.* 7	7.01[d]	*	8.83[d]	*	*
	5 *vs.* 7	5.68[c]	*	*	*	*
	6 *vs.* 7	6.03[d]	*	7.66[d]	*	*

[a] * indicates values less than 1.0.
[b] See Table II for legend.
[c] Indicate significance at the 0.05 level.
[d] Indicates significance at the 0.01 level.

TABLE VII.—CONCENTRATION MEANS OF THE VARIOUS ACID-SOLUBLE PHOSPHATE COMPOUNDS DETERMINED IN THE ILEUM STRIPS OF RAT INTESTINES SUBJECTED TO INITIAL ANAEROBIOSIS AND SUBSEQUENT AEROBIOSIS[a,b]

Treatment Number	Mg. % Phosphorus (Wet Weight of Tissue)				
	T. A. S. P.–P.	I. P.–P.	Cr. P.–P.	A. T. P.–P.	~ P.–P.
1	142.27 ± 10.40	25.28 ± 5.17	8.44 ± 1.54	5.64 ± 0.79	14.08 ± 0.92
2	126.87 ± 10.00	26.89 ± 2.58	6.96 ± 1.02	5.24 ± 1.18	12.19 ± 2.04
3	136.05 ± 11.32	27.28 ± 2.62	6.66 ± 0.80	4.57 ± 1.18	11.24 ± 1.68
4	104.10 ± 16.26	27.32 ± 3.32	7.10 ± 0.83	3.95 ± 0.65	11.06 ± 2.42
5	128.57 ± 6.03	26.21 ± 3.85	9.89 ± 3.22	5.39 ± 1.97	15.29 ± 1.74
6	124.60 ± 28.22	25.77 ± 1.30	5.60 ± 0.85	4.96 ± 2.11	10.57 ± 2.42
7	153.77 ± 20.65	27.55 ± 0.92	11.29 ± 0.96	6.45 ± 2.09	17.73 ± 1.34

[a] Treatment number corresponds to those in Table III.

[b] $S.E.M. = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

in strips subjected to the anoxic period. Figure 6 and 7 illustrate the concentration patterns shown by the various high-energy phosphate compounds of both the duodenal-jejunum and the ileum strips.

The analysis of variance for I. P.–P., A. T. P.–P. and energy-rich phosphate phosphorus did not show significant changes with the treatments, but the trends indicate that the strips maintained in oxygenated Kreb-Henseleit solution containing thyroxin increased in their amounts of energy-rich phosphate compounds. These trends are similar to those found for the duodenal-jejunum strips.

DISCUSSION

In the substrate depletion studies, although no exogenous substrate was present, the breakdown of the mucosa might have been responsible for the failure to obtain significant reductions in the labile phosphates of the smooth muscle. Incubation in a muscle bath for thirty minutes or more caused the mucosa to loosen and separate from the smooth muscle. This disintegration might have caused the release of enough metabolites into the bathing fluid to supply the intestinal smooth muscle with sufficient substrate to prevent the depletion of the energy-rich phosphate. In the work reported by Furchgott and Shorr (15) smooth muscle, longitudinal and circular, of dog intestine which had been stripped of its mucosa was used for anoxic incubation and subsequent phosphate assay. This technique would appear to eliminate the influence of intestinal mucosa degeneration.

With regard to the studies made by Furchgott and Shorr (15), the values which they obtained for anoxic treatment of rabbit intestines showed that anoxia decreased the amounts of energy-rich phosphates in the intestine; these strips, however, were not contracting against tension and thus not duplicating pharmacological testing conditions. The method for suspending the intestinal strips in this study was concerned only with the contractions of the longitudinal smooth muscle. Phosphate analyses, however, were conducted on both longitudinal and circular muscle. Attempts were made to analyze intestinal strips which had been mounted in a manner used to record longitudinal and circular muscle contractions, but the manipulations required for dismounting the tissue and preparation for assay caused trauma which would seem to invalidate the results.

The recent publications by Minz, *et al.* (21), and Thibault (22) reported that the motility of rabbit intestine was increased when intestinal strips were allowed to bathe in physiological solutions containing thyroxin for a period of one hour. The hypothesis proposed was that thyroxin is converted into "active thyroxin" which is concerned with the synthesis of coenzyme A and caused increased formation of acetylcholine. Little work of a chemical nature has been done to confirm or deny this hypothesis, but from the studies reported in this paper on rat intestine, thyroxin appeared to increase the amounts of energy-rich phosphate of the muscularis of intestinal strips.

CONCLUSIONS

1. The endogenous substrate depletion of rat ileum strips appeared to reduce the amounts of creatine phosphate, adenosine triphosphate, and energy-rich phosphate; and the addition of exogenous substrate after a ninety-minute depletion appeared to increase the amounts of these phosphates.

2. Anoxic conditions appeared to decrease the amounts of creatine phosphate, adenosine triphosphate, and energy-rich phosphate of duo-

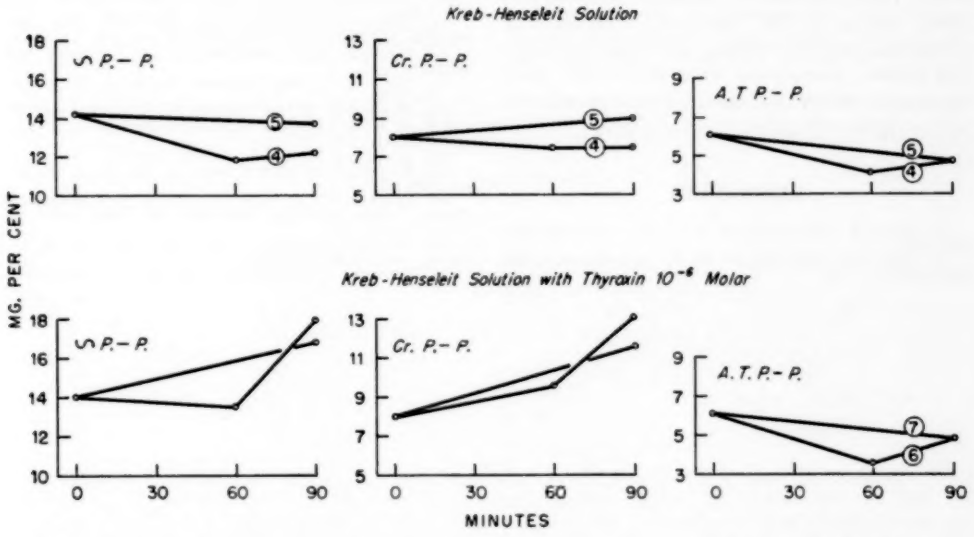

Fig. 6.—Concentration means, mg. %, of the energy-rich phosphate compounds in rat duodenal-Jejunum strips subjected to anaerobiosis and subsequent aerobiosis in Kreb-Henseleit solution with and without thyroxin vs. time of incubation.

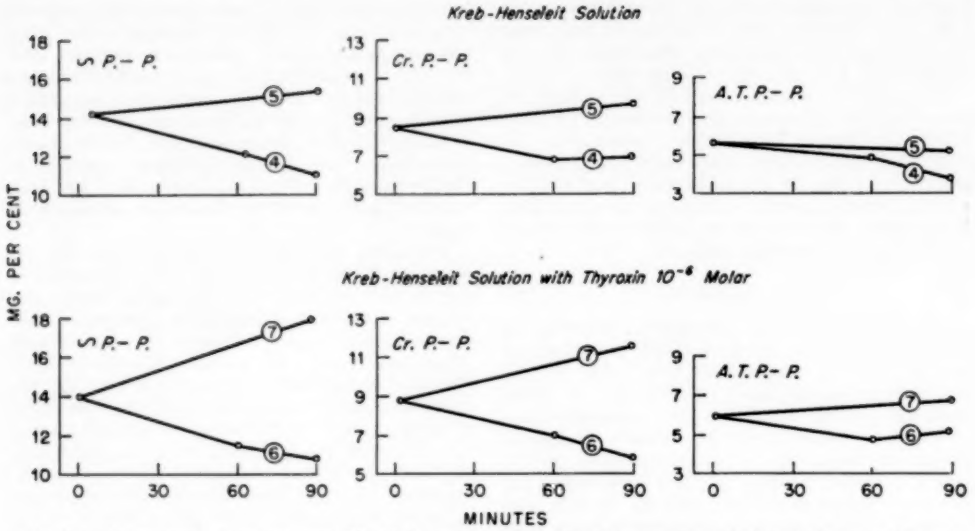

Fig. 7.—Concentration means, mg. %, of the energy-rich phosphate compounds in rat ileum strips subjected to anaerobiosis and subsequent aerobiosis in Kreb-Henseleit solution with and without thyroxin vs. time of incubation.

denal-jejunum strips of rat intestine when suspended in Kreb-Henseleit bicarbonate solution with glucose, 100 mg. %. Ileum strips of rat intestine appeared to show similar changes under the same conditions.

3. The presence of thyroxin, 10^{-6} molar final concentration, in Kreb-Henseleit bicarbonate solution with glucose, 100 mg. %, appeared to increase the amounts of creatine phosphate and energy-rich phosphate in duodenal-jejunum and ileum strips of rat intestine. Significantly greater amounts of creatine phosphate and total acid-soluble phosphates were found for ileum strips mounted in this bathing fluid aerated with 95% oxygen–5% carbon dioxide mixture.

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The Activity and Toxicity of Ampules Amytal Sodium and Seconal Sodium in Aqueous Polyethylene Glycol 200*,†

By E. E. SWANSON, R. C. ANDERSON, P. N. HARRIS, and C. L. ROSE‡

THE PHARMACOLOGIC properties, including the anesthetic and lethal doses of Amytal Sodium and Seconal Sodium by various methods of administration in different animals, were previously reported from this laboratory (1–11). The present study was undertaken to determine whether or not the activity and toxicity of Amytal Sodium and Seconal Sodium would be changed by 60 and 50 per cent, respectively, of polyethylene glycol 200.

EXPERIMENTAL

Rats were used. Single doses of barbiturates were injected into the tail vein. The median anesthetic dose (AD_{50}), determined by absence of response to tail pinching, was found to be 75.0 ± 3.5 mg. per Kg. for Amytal Sodium and 39.0 ± 1.2 mg. per Kg. for Seconal Sodium. The median lethal dose (LD_{50}) of both barbiturates was approximately twice as large as the AD_{50}, making a ratio of 2:1 as shown in Table I. This is the therapeutic index for most of the barbiturates now in clinical use.

The next series of experiments was directed to determine the subacute toxicity of intravenous injection in rabbits (ear vein). The dose schedule, as indicated in Table II, was one a day, five days a week. The entire period of the experiment lasted four weeks. In addition, a control series of rabbits was injected with 60% polyethylene glycol 200 alone in the same volume as above, but without the barbiturate.

Table II presents the results obtained with Amytal Sodium in 60% polyethylene glycol 200, with Seconal Sodium in 50% polyethylene glycol 200, and with the solvent alone. Only one animal of the twelve that received Amytal Sodium succumbed. The cause of death was not evident. All survivors were sacrificed and submitted for necropsy. Microscopic examination of the heart, lungs, liver, spleen, kidneys, thymus, thyroid, and adrenal glands was made of all animals. As recorded in Table II, there was a region of cortical necrosis and calcification of the adrenal gland in one animal receiving the Amytal Sodium in 60% polyethylene glycol 200; and a region of necrosis involving the cortex and perhaps the medulla in another animal. Neither of these lesions appeared to be caused by the drug. All other organs of these rabbits were normal.

Although several animals receiving Seconal Sodium in 50% polyethylene glycol 200 died, there was no correlation with the size of the dose administered. For example, there was one death out of three on 10 mg. per Kg. and one of three on 15 and 20 mg. per Kg. Similarly, death occurred in two out of three rabbits on 0.1 cc. per Kg. of the solvent (60% polyethylene glycol 200), one out of three with 0.3 cc. per Kg.; and one out of three with 0.4 cc. per Kg. As is to be noted in Table II, there were erythrocytes in the renal tubules of one animal on the smallest dose of Seconal Sodium, which did not cause death for the entire experimental period of four weeks. The same is true with two animals that received 15 mg. per Kg. The rabbits that died on the 10 and 20 mg. per Kg. doses had some pulmonary edema and diarrhea, but no red blood cells in the renal tubules. With polyethylene glycol 200 alone, one of the two rabbits that succumbed to the smallest dose developed diarrhea, emaciation, and dehydration, the other, bronchopneumonia. With a dose of 0.2 cc. per Kg. one rabbit died with diarrhea and

TABLE I.—ACUTE TOXICITY AND ANESTHETIC DOSE OF AMPULES OF AMYTAL SODIUM IN 60% POLYETHYLENE GLYCOL 200 AND SECONAL SODIUM IN 50% POLYETHYLENE GLYCOL 200, IN RATS (INTRAVENOUSLY)

Barbiturate	Dosage, Mg./Kg.	Anesthesia Total Number	No. Died Total Number	AD_{50} ± S. E., Mg./Kg.	LD_{50} ± S. E., Mg./Kg.
Amytal Sodium	62	2/8	...	75.0 ± 3.5	...
	70	7/21	...		
	80	15/24	...		
	100	...	1/24	...	136.0 ± 3.9
	125	...	7/24		
	160	...	20/24		
Seconal Sodium	33	5/24	...	39.0 ± 1.2	...
	40	17/32	...		
	45	19/24	...		
	62	...	5/24	...	73.1 ± 2.4
	70	...	10/24		
	80	...	16/24		

* Received February 24, 1953, from the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.

† 'Seconal Sodium' is a trade mark identifying the Eli Lilly and Company brand of secobarbital sodium and 'Amytal Sodium' is a trade mark identifying the Eli Lilly and Company brand of amobarbital sodium.

‡ The authors are indebted to Dr. K. K. Chen and Miss Eva Sommermeyer for their helpful suggestions and criticisms of this report.

TABLE II.—SUBACUTE TOXICITY OF AMPULES OF AMYTAL SODIUM AND SECONAL SODIUM IN RABBITS (INTRAVENOUSLY)

Drug	Daily Dose	No. Died / No. Used	Pathological Findings
Amytal Sodium 5% in 60% Polyethylene Glycol 200	10 Mg./Kg. (0.2 Cc./Kg.)	0/4	3 Normal; 1 region of cortical necrosis and calcification of adrenal gland
	20 Mg./Kg. (0.4 Cc./Kg.)	0/4	3 Normal; 1 region of necrosis involving cortex and perhaps the medulla of the adrenal
	40 Mg./Kg. (0.8 Cc./Kg.)	1/4	3 Normal; 1 died after six doses—cause of death was not evident
Seconal Sodium 5% in 50% Polyethylene Glycol 200	5 Mg./Kg. (0.1 Cc./Kg.)	0/3	2 Normal; 1 R. B. C. in collecting tubules
	10 Mg./Kg. (0.2 Cc./Kg.)	1/3	2 Normal; 1 died after 20 doses, pulmonary edema and diarrhea
	15 Mg./Kg. (0.3 Cc./Kg.)	1/3	1 Died after 20 doses, cause of death was not evident. 2 R. B. C. in collecting tubules
	20 Mg./Kg. (0.4 Cc./Kg.)	1/3	1 Died after 17 doses, pulmonary edema and diarrhea; 1 normal; 1 mild renal injury
60% Polyethylene Glycol 200	(0.1 Cc./Kg.)	2/3	1 Died after 19 doses, emaciation, dehydration and diarrhea; 1 died after 17 doses, bronchopneumonia; 1 normal
	(0.2 Cc./Kg.)	0/3	2 Normal; 1 slight calcification in renal tubules
	(0.3 Cc./Kg.)	1/3	1 Died after 16 doses, malnutrition and diarrhea; 1 normal; 1 fatty metamorphosis of the liver
	(0.4 Cc./Kg.)	1/3	1 Died after 15 doses, cause of death was not evident; 2 normal

malnutrition. One surviving rabbit in this group showed fatty metamorphosis of the liver.

While it could not be proved, it appeared that our rabbit colony was suffering from an epidemic of some infection, resulting in pathological manifestations during the period when the Seconal Sodium and 60 per cent polyethylene glycol 200 experiments were conducted. The occurrence of erythrocytes in the renal tubules probably was not attributable to polyethylene glycol 200 because it has been observed in several other series of experiments with different drugs and in untreated animals. In view of the fact that the frequency and the severity of the deleterious effects bear no relation to the size of the dose, it was not likely that either the barbiturate or the solvent was responsible for them. There was some edema in the ears where leaking into the subcutaneous tissue occurred.

Both the Amytal Sodium and Seconal Sodium solutions in water and in polyethylene glycol 200 were tested for irritation in rabbits by injection into the sacrospinalis muscles. Control studies were also made with 60% polyethylene glycol 200. The doses varied from 0.1 to 0.5 cc. Three animals were employed for each dose. All rabbits were sacrificed seventy-two hours after injection, and the injected muscles examined grossly and microscopically.

At necropsy, regions of necrosis of the muscle were visible to the naked eye and were measured for length, width, and depth. The products of the measurements expressed in cu. cm. approximately represent the range of the volume of necrosis and the degrees of irritation arbitrarily rated, as shown in Table III. As expected, the smaller the dose, the less the irritation. Polyethylene glycol 200, 60% in water alone was relatively less irritating than Amytal Sodium in 60% polyethylene glycol 200, volume for volume. Amytal Sodium in water appeared to be slightly more irritating than in 60% polyethylene glycol 200. The same was true for Seconal Sodium. Polyethylene glycol 200, 50% in water alone was relatively less irritating than Seconal Sodium in 50% polyethylene glycol 200 volume for volume. Seconal Sodium in water appeared to be slightly more irritating than in 50% polyethylene glycol 200. Microscopically, all sections of necrotic fibers looked alike.

TABLE III.—IRRITATION IN RABBITS FOLLOWING INTRAMUSCULAR INJECTION

Preparation	Dose, Cc.	Irritation: Necrotic Value Range, cu. cm.	Irritation: Rating
Amytal Sodium 5% in water	0.5	1.4–1.8	Severe
	0.25	1.2–1.8	Severe
	0.1	0.2–0.9	Mild
Amytal Sodium 5% in 60% Polyethylene Glycol 200	0.5	1.08–1.47	Moderate
	0.25	0.5–0.9	Mild
	0.1	0.04–0.3	Slight
Polyethylene Glycol 200, 60% in Water	0.5	1.5–1.8	Severe
	0.25	0.4–0.5	Mild
	0.1	0.2–0.4	Slight
Seconal Sodium 5% in Water	0.5	1.5 or more	Severe
	0.25	1.0–1.5	Moderate
	0.1	0.4–1.0	Mild
Seconal Sodium 5% in 50% Polyethylene Glycol 200	0.5	1.5 or more	Severe
	0.25	0.5–1.0	Mild
	0.1	0.5–1.0	Mild
Polyethylene Glycol 200, 50% in Water	0.5	0.5–1.0	Mild
	0.25	0.3–0.5	Slight
	0.1	0.2–0.4	Slight

SUMMARY

1. The present study was undertaken to determine whether or not the activity and toxicity of Amytal Sodium and Seconal Sodium would be changed by 60 and 50 per cent, respectively, of polyethylene glycol 200.

2. Amytal Sodium in 60 per cent polyethylene glycol 200 and Seconal Sodium in 50 per cent polyethylene glycol 200 showed approximately the same potency and toxicity as aqueous solutions of these barbiturates. The median anesthetic dose (AD_{50}) by vein in rats was 75.0 ± 3.5 mg. per Kg. for Amytal Sodium and 39.0 ± 1.2 mg. per Kg. for Seconal Sodium. The median lethal doses (LD_{50}) were approximately twice as large as the AD_{50}'s. This is a ratio of nearly 2:1, which is common to most barbiturates now in clinical use.

3. In the subacute toxicity experiments both barbiturates in polyethylene glycol 200 produced no obvious pathological changes.

4. When injected intramuscularly in rabbits, the aqueous solutions of the two barbiturates produced more irritation in the tissues than solutions of the two compounds in polyethylene glycol 200.

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Experiments with Khellin. V.[1] The Structure of Desmethylisokhellin*

By HAMED ABU-SHADY† and TAITO O. SOINE‡

Chemical evidence is presented to show that desmethylisokhellin is 5-hydroxy-6-methoxy-2-methylfuro(2′,3′,7,8)chromone as had been predicted previously. Several new compounds incidental to the proof of structure have been prepared and the concept of the structure of desmethylisokhellin is supported by the infrared spectrum which shows a band attributed to a bonded hydroxyl group.

IN A RECENT communication the preparation of the phenol, desmethylisokhellin, has been described (1). Under the influence of 48 per cent hydrobromic acid, khellin undergoes a series of transformations to yield the monophenol (desmethylisokhellin) as the main component of the reaction mixture. When methylated in the usual way by the methyl iodide-potassium carbonate method, desmethylisokhellin was converted to isokhellin, the structure of which had been established in an earlier communication (2). On the other hand, desmethylisokhellin would not methylate with diazomethane, a fact indicative of the low acidity of the phenolic group which was believed to be hydrogen bonded with an adjacent carbonyl group. This concept of the structure of desmethylisokhellin was supported by the infrared spectrum which showed a band at 3,100 cm.$^{-1}$ that was attributed to a bonded hydroxyl group.

In this paper, however, chemical evidence is presented to prove beyond doubt that desmethylisokhellin is II and not I. Desmethylisokhellin was converted to its benzyl ether (III) by the

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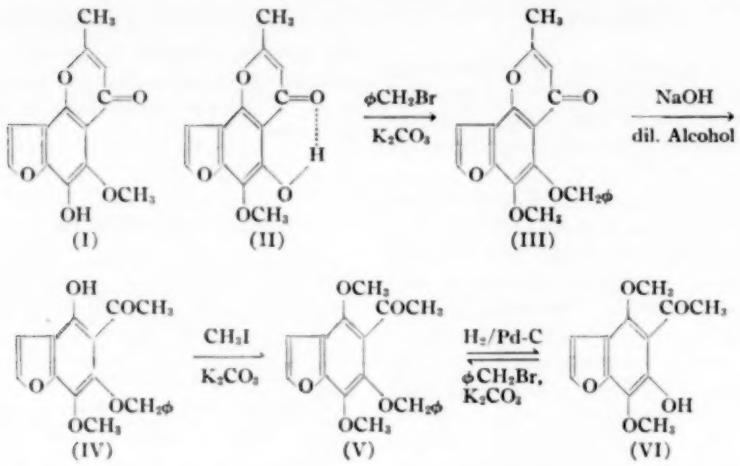
‡ The authors wish to acknowledge the generous financial assistance of the Egyptian Education Bureau and the American Foundation for Pharmaceutical Education. Thanks are also due to the Smith, Kline and French Company for a gift of khellin.



action of benzyl bromide and potassium carbonate. The benzyl ether was then treated with sodium hydroxide solution to hydrolyze the γ-pyrone ring. The resulting *o*-hydroxyketone (IV) was characterized as its 2,4-dinitrophenylhydrazone and was methylated with methyl iodide and potassium carbonate to yield benzylkhellinone (V). This derivative of khellinone was found to be identical with a sample prepared from authentic khellinone. Furthermore, benzylkhellinone (V) was hydrogenolyzed to yield khellinone (VI) which was identical with authentic khellinone (mixed m. p.).

The ultraviolet spectra of khellin and isokhellin together with the corresponding desmethyl compounds are shown in Figs. 1 and 2.

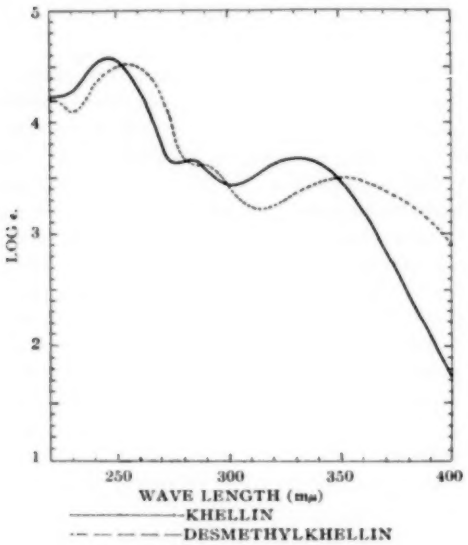


FIGURE 1.

The maxima of all compounds are presented in Table I. It is interesting to note the 8 mμ shift toward the longer wave length in passing from the methyl ether to the phenol in each case in the first maximum.

TABLE I.—ULTRAVIOLET ABSORPTION DATA[a]

Compound	Ethanol, 95% λMax., mμ	Log ε
Khellin[b]	247	4.57
	281	3.67
	331	3.67
Desmethylkhellin	255	4.52
	~287.5	...
	353	3.49
Isokhellin	243	4.47
	319	3.66
Desmethylisokhellin	251	4.45
	344	3.45

[a] The authors wish to acknowledge the assistance of H. J. Rhodes of this laboratory in the checking and reproduction of the ultraviolet data.

[b] These values agree closely with those of Bailey, S. D., Geary, P. A., and deWald, A. E., THIS JOURNAL, **40**, 280 (1951).

EXPERIMENTAL

5-Hydroxy-6-methoxy-2-methylfuro(2′,3′,7,8)-chromone (Desmethylisokhellin, II).—This phenol (m. p. 177°) was prepared from khellin by the action of 48% hydrobromic acid (sp. gr. 1.49) according to the method of Abu-Shady and Soine (1).

5-Benzyloxy-6-methoxy-2-methylfuro(2′,3′,7,8)-chromone (Benzyldesmethylisokhellin, III).—Desmethylisokhellin (10 Gm.) was refluxed for seventy hours with benzyl bromide (7 cc.), anhydrous potassium carbonate (20 Gm.), and acetone (200 cc.). The reaction mixture was filtered and evaporated to a small volume to yield 6.5 Gm. of slightly yellow crystals. These crystals were washed with ether and then crystallized three times from methyl alcohol to give an analytical sample, m. p. 140°.

Anal.—Calcd. for $C_{20}H_{16}O_5$: C, 71.42; H, 4.80. Found: C, 71.40; H, 4.94.

5-Acetyl-6-benzyloxy-4-hydroxy-7-methoxycoumarone (IV).—Benzyldesmethylisokhellin (5 Gm.) was refluxed for thirty minutes with a solution containing 10 Gm. of sodium hydroxide in a mixture

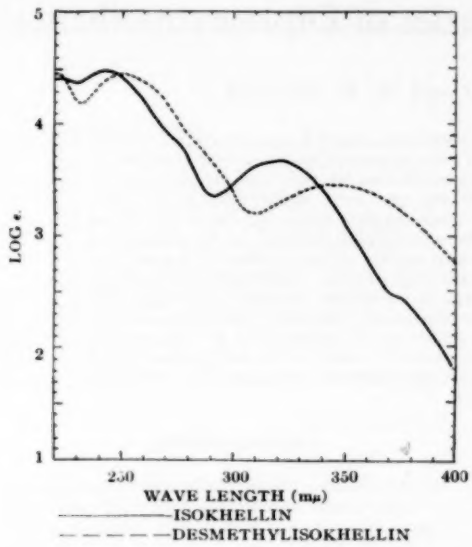


FIGURE 2

of 50 cc. of alcohol and 50 cc. of water. The refluxed mixture was cooled, carefully acidified with acetic acid, and then extracted thoroughly with ether. The ethereal solution was dried over anhydrous sodium sulfate and then evaporated to dryness to yield a yellow liquid that failed to crystallize. In order to obtain an analysis on this compound a small portion of it was converted in the usual way to the 2,4-dinitrophenylhydrazone, m. p. 156.5–157°, which was then analyzed.

Anal.—Calcd. for $C_{24}H_{20}O_8N_4$: C, 58.53; H, 4.09. Found: C, 58.83; H, 4.30.

5-Acetyl-6-benzyloxy-4,7-dimethoxycoumarone (Benzylkhellinone, V).—A portion of the uncrystallizable, yellow liquid from the preceding experiment was subjected to the usual methyl iodide-potassium carbonate treatment in acetone and yielded a colorless, crystalline product, m. p. 86°. On comparison with a sample of benzylkhellinone prepared from khellinone by the action of methyl iodide and potassium carbonate in acetone, it was found that they were identical. A mixed melting point with the known benzylkhellinone failed to depress the melting point.

Anal.—Calcd. for $C_{19}H_{18}O_5$: C, 69.93; H, 5.56. Found: C, 70.23; H, 5.85.

5-Acetyl-4,7-dimethoxy-6-hydroxycoumarone (Khellinone, VI).—A small sample of benzylkhellinone in alcohol was hydrogenolyzed in the usual way (2) in the presence of a palladium-on-charcoal (10%) catalyst. The alcoholic solution was then freed from the catalyst by filtration and evaporated on a steam bath to dryness. The residue was taken up in 10% sodium hydroxide solution and the yellow alkaline solution was then shaken successively with small volumes of ether to remove the unchanged benzyl ether. The alkaline solution, after being freed of ether on a steam bath, was acidified with dilute hydrochloric acid and again extracted completely with ether. The combined ethereal extracts were washed with water, dried, and concentrated to a small volume. The yellow crystals that separated on cooling melted at 95° and were identified as khellinone on the basis of a mixed melting point determination with an authentic sample of khellinone prepared from khellin by hydrolysis using the method of Spaeth and Gruber (3).

Ultraviolet Spectra.—The ultraviolet absorption spectra reproduced in Table I were determined in alcoholic solution with a Beckman Model DU Quartz Spectrophotometer; $\epsilon = 1/c \log_{10} I_0/I$ where c represents moles per liter.

SUMMARY

1. A chemical proof has been established to show that desmethylisokhellin is, indeed, 5-hydroxy-6-methoxy-2-methylfuro(2′,3′,7,8)chromone as had previously been predicted.

2. Several new compounds incidental to the proof of structure have been prepared.

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Antibacterial Activity of a Series of Diphenylmethanes*

By H. J. FLORESTANO and M. E. BAHLER

Thirty-eight derivatives of diphenylmethane were examined for antibacterial activity against a variety of organisms. Activity *in vitro* appeared more or less specific for Gram-positive bacteria, twenty-three derivatives being bactericidal for this type in concentrations ranging from 0.004 to 4.0 mg. per cent, while only seven compounds were active against the respective Gram-negative organisms at levels of 0.5 to 4.0 mg. per cent. Activity of the diphenylmethanes seemed to be dependent upon both hydroxy and halogen group substitution in the parent structure, the position of these groups being of considerable importance. Substitution of various other groupings are discussed in an attempt to correlate chemical structure with activity. Antibacterial properties of the diphenylmethanes were markedly reduced in the presence of serum. Although the majority of compounds in the present series were found highly bactericidal for streptococci, none of the derivatives tested showed any marked protective action against experimental streptococcal infection in mice.

CONSIDERABLE attention has been given in past years to the investigation of the germicidal properties of phenols (1). Bisphenols have also been extensively studied (2–5). In the latter group, 2 derivatives of the class containing the $—CH_2—$ linkage have been shown to possess marked antimicrobial activity, i. e., bis(2-hydroxy-5-chlorophenyl)methane or G-4, and bis(2 - hydroxy - 3,5,6 - trichlorophenyl)methane, more commonly known as G-11 or hexachlorophene. The first has been used in the textile industry as a mildew preventive (6), while G-11 incorporated in soaps and other products has proved highly effective as a skin disinfectant (7–9).

During an investigation of various bisphenols, a number of new derivatives of diphenylmethane were synthesized in our laboratory and tested for antimicrobial properties. The tuberculocidal activities of some of these compounds have already been described (10). The present report deals with a study of the general antibacterial properties of the compounds, in which comparison has been made with known related derivatives in an attempt to correlate activity with chemical structure.

It has been reported (7) that the antibacterial activity of G-11 is reduced in the presence of serum, and that this effect can be duplicated by egg albumin or serum globulin (11). In order to determine whether this action of protein is specific for diphenylmethanes, a similar study was undertaken to include members of the present series. Observations relative to the efficacy of some of the compounds in experimental streptococcal infections are also presented.

EXPERIMENTAL

In vitro Study.—Activities of the various derivatives were tested against the following organisms: *Streptococcus pyogenes* (C203), *Streptococcus agalactiae*, *Staphylococcus aureus* (Barlow),[1] *Escherichia coli*, *Shigella gallinarum*, and *Salmonella pullorum*. Brain heart infusion broth (Difco) was used as the test medium for the C203 strain of streptococcus and proteose-peptone No. 3 broth (Difco) for *Streptococcus agalactiae*. In experiments with *Staphylococcus aureus* the synthetic medium described by Straus, *et al.* (12), was employed, and that of Sahyun, *et al.* (13), used for *E. coli*, *Shigella gallinarum*, and *Salmonella pullorum*. For optimum growth of the *Shigella* and *Salmonella* strains the medium was enriched with casein hydrolysate (1% Casamino acids, Difco).

Because of the low solubility of most of the derivatives in water, stock solutions were prepared by dissolving the respective compounds in 95% ethyl alcohol to a concentration of 500 mg. %. Subsequent dilutions were made aseptically in tubes containing the test medium so that each compound was assayed at a maximum of 4.0 mg. %, with lower concentrations decreasing by halves. Each tube contained a final volume of 5.0 cc. of medium plus drug at the various dilutions. The inoculum consisted of 0.1 cc. of a suitably diluted eighteen-hour culture of test organism so that the initial number of bacteria averaged about 500 per cc. The tubes were incubated at 37° and examined for visible growth at twenty-four-hour intervals for three days. While controls on the diluent were included with each test, it was observed in repeated runs that the concentration of ethyl alcohol necessary to inhibit growth of the test strains greatly exceeded that present in the initial tube of the dilution series. Compounds showing complete inhibition were tested for bactericidal properties by subculturing into 10 cc. of plain medium, inoculating the largest amount of drug-broth possible (0.1 to 1.0 cc.) without introducing an inhibitory concentration *per se*. All subcultures were incubated at 37° for a maximum of three days.

* Received January 6, 1953, from the Research Department, Pitman-Moore Company, Division of Allied Laboratories, Inc., Indianapolis, Indiana.

[1] The older terminology of *Staphylococcus aureus* has been retained here for what is now described as *Micrococcus pyogenes* var. *aureus* in "Bergey's Manual of Determinative Bacteriology" (6th ed.).

TABLE I.—ANTIBACTERIAL ACTIVITIES OF DIPHENYLMETHANE DERIVATIVES

No.	Compound[a]	Minimum Bactericidal Concentration in Mg. % After 72 Hours Incubation					
		Strep. pyogenes (C203)	*Strep. agalactiae*	*Staph. aureus*	*E. coli*	*Sh. gallinarum*	*Sal. pullorum*
1	Diphenylmethane	4.0	4.0	>4.0	>4.0	>4.0	>4.0
2	Bis(4-aminophenyl)methane	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
3	Bis(2-hydroxyphenyl)methane	2.0	4.0	>4.0	>4.0	>4.0	>4.0
4	Bis(4-chlorophenyl)methane	0.25	0.25	>4.0	>4.0	>4.0	>4.0
5	2,4′-Dichlorodiphenylmethane	0.25	0.25	>4.0	>4.0	>4.0	>4.0
6	Bis(2-hydroxy-5-bromophenyl)methane	0.031	0.125	0.063	0.5	0.5	1.0
7	Bis(2-hydroxy-5-chlorophenyl)methane	0.25	0.25	0.25	1.0	0.5	1.0
8	5,3′-Dihydroxy-2,4′-dichlorodiphenylmethane	0.25	0.5	1.0	4.0	2.0	4.0
9	Bis(2-hydroxy-3,5-dichlorophenyl)methane	0.125	0.25	0.125	>4.0	>4.0	>4.0
10	Bis(2-hydroxy-3,5,6-trichlorophenyl)methane	0.004	0.063	0.016	0.5	1.0	1.0
11	Bis(2,4-dihydroxy-5-chlorophenyl)methane	1.0	>4.0	1.0	>4.0	>4.0	>4.0
12	Bis(2-methoxy-5-chlorophenyl)methane	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
13	Isomeric mixture[b]	0.016	0.063	0.063	4.0	2.0	4.0
14	Bis(2-hydroxy-4 or 6-methyl-5-chlorophenyl)-methane[c]	...	0.125	2.0	>4.0	...	...
15	Disodium 2,2′-methylenebis(4-chlorophenyl-1-oxyacetate)	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
16	2,2′-Dihydroxy-3-hydroxymethyl-5,5′-dichlorodiphenylmethane	0.5	1.0	1.0	>4.0	4.0	4.0
17	Bis(2-hydroxy-3-hydroxymethyl-5-chlorophenyl)methane	1.0	2.0	4.0	>4.0	4.0	>4.0
18	Bis(2-hydroxy-3-iodo-5-chlorophenyl)-methane	0.031	0.25	0.25	>4.0	>4.0	>4.0
19	2,2′-Ethylidenebis(4-chloro-6-iodophenol)	0.016	0.063	0.063	4.0	4.0	>4.0
20	2,2′-Benzalbis(4-chloro-6-iodophenol)	0.008	0.063	0.063	>4.0	>4.0	>4.0
21	2,2′-6′,2″-Dimethylenetris(4-chlorophenol)	0.016	0.063	0.063	>4.0	>4.0	>4.0
22	Bis(3-carboxy-4-hydroxyphenyl)methane	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
23	Bis(2-hydroxy-5-nitrophenyl)methane	0.5	2.0	2.0	>4.0	>4.0	>4.0
24	Bis(2-hydroxy-5-arsonophenyl)methane	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
25	Bis(2-methyl-4-hydroxy-5-isopropylphenyl)-methane	0.125	0.063	2.0	>4.0	>4.0	>4.0
26	2,2′-Dihydroxy-3-isopropyl-5,5′-dichloro-6-methyldiphenylmethane	0.063	0.063	0.016	>4.0	0.5	1.0
27	Bis(2-hydroxy-3-isopropyl-5-chloro-6-methylphenyl)methane	0.016	0.031	0.031	>4.0	>4.0	>4.0
28	2,4′Dihydroxy-6,2′-dimethyl-3,5′-di-isopropyl-5-chlorodiphenylmethane	0.063	0.5	>4.0	>4.0	>4.0	>4.0
29	Bis(2-hydroxy-5-*tert.*-butylphenyl)methane	0.063	0.125	1.0	>4.0	>4.0	>4.0
30	2,2′-Dihydroxy-3-isopropyl-5-chloro-5′-*tert.*-butyl-6-methyldiphenylmethane	0.008	0.031	>4.0	>4.0	>4.0	>4.0
31	2,2′-Dihydroxy-3-isopropyl-3′-iodo-5-chloro-5′-*tert.*-butyl-6-methyldiphenylmethane	0.016	0.5	1.0	4.0	>4.0	>4.0
32	Bis(2-hydroxy-5-*tert.*-amylphenyl)methane	0.063	0.125	1.0	>4.0	>4.0	>4.0
33	Bis-dicyclohexylamine salt of bis(2-hydroxy-5-chlorophenyl)methane	0.5	1.0	2.0	2.0	1.0	2.0
34	1,1′-Bis(2-hydroxynaphthyl)methane	0.5	0.5	>4.0	>4.0	>4.0	>4.0
35	Methyl-bis(2-hydroxy-5-chlorophenyl)-methane	0.25	0.25	0.25	4.0	2.0	2.0
36	1,1-Di-4-chlorodiphenyl-2,2,2-trichloroethane	0.125	0.25	>4.0	>4.0	>4.0	>4.0
37	2,2′-Benzalbis(4-chlorophenol)	0.125	0.125	0.125	2.0	2.0	2.0
38	3,3′-Dichlorobenzohydrol	1.0	2.0	4.0	>4.0	4.0	>4.0
39	Bis(4-chlorophenylmercapto)methane	0.25	>4.0	>4.0	>4.0	>4.0	>4.0
40	Phenol	...	128	256	128	...	...

[a] Compounds Nos. 1 and 2 were obtained from Eastman Kodak Co.; Nos. 7 and 10 from Givaudan-Delawanna, Inc.; No. 22 from Heyden Chemical Corp.; No. 36 from Merck and Co., Inc. Nos. 3, 4, 5, 6, 9, 12, 13, 21, 23, 25, and 34 are known compounds which were prepared in this laboratory by H. E. Faith for purpose of comparison. All other compounds were synthesized here as new and undescribed derivatives of diphenylmethane as far as could be determined from a search of the literature.

[b] No. 13 is a mixture of bis(2-hydroxy-4-methyl-5-chlorophenyl)methane and bis(2-hydroxy-6-methyl-5-chlorophenyl)-methane.

[c] No. 14 is one of the components of No. 13 isolated in pure form.

Experiments dealing with the effect of protein on the *in vitro* activities of diphenylmethanes were performed by the same procedure described above, using the strain of *Staphylococcus aureus* as the test organism. Solutions of bovine albumin (fraction V), sterilized by Seitz filtration, were added to the medium aseptically at a concentration of 1%. A parallel series containing drug dilutions without albumin was included in each run.

In vivo Study.—The relative activities of 11 of the diphenylmethane derivatives were determined against streptococcal infection in mice. Selection of drugs and dosages employed was based upon activity shown *in vitro* and toxicity data obtained in mice (10). Animals (CFW) weighing 20 Gm. each were used throughout. Infection was by the intraperitoneal route, 0.5 cc. of a 1:500,000 dilution of an eighteen-hour blood broth culture of the C203

strain of streptococcus serving as the inoculum. Virulence titration indicated that such an inoculum contained 100 MLD. The animals were treated once daily for three successive days, the initial dose being administered immediately after infection. All of the compounds were given by the oral route; several derivatives were in addition tested subcutaneously. Suspensions of drug in 10% gum acacia solution were used *per os* and aqueous solutions of the sodium salts for treatment parenterally. Mice were observed for fifteen days following infection. Animals dying within the period of test were autopsied and heart blood cultured in suitable media.

RESULTS

In vitro data obtained with diphenylmethane and 38 derivatives showed that the compounds were, in general, far more active against the Gram-positive bacteria than against the Gram-negative species tested (Table I). The limited solubilities of the derivatives did not allow for determination of the exact end points for most of them against the Gram-negative bacteria, but only seven compounds (Nos. 6, 7, 8, 10, 33, 35, and 37) possessed bactericidal activity for the three species studied at 4.0 mg. % or less. On the other hand, 23 derivatives were bactericidal for each of the Gram-positive organisms within the range tested.

Activity of the diphenylmethanes appeared dependent not only upon hydroxy and halogen group substitution in the parent structure but upon position as well (compounds No. 1–11). In the basic structure, bis(2-hydroxy-5-halophenyl)methane, bromine substitution (No. 6) resulted in greater activity than chlorine (No. 7) against Gram-positive organisms, while bactericidal action on Gram-negatives remained equal. Substitution of an additional pair of chlorine atoms in the 3,3′-positions (No. 9) showed no advantage over the dichloro derivative (No. 7) in so far as Gram-positive activity was concerned, but activity against Gram-negative organisms was greatly reduced. However, upon further substitution of chlorine in the 6,6′-positions (No. 10) Gram-negative activity comparable to that of No. 7 was again indicated, with decidedly superior activity being shown against the Gram-p sitive organisms. The significance of halogen in addition to hydroxy groups on the ring for over-all activity was further apparent from the results obtained with compounds No. 12, 22, 23, 24, 25, 29, 32, 34, and 38. Compared with Nos. 6, 7, 8, and 10, these nine derivatives showed no demonstrable activity against Gram-negative organisms at the maximum concentration tested, with the exception, however, of No. 38 which was bactericidal for *Shigella gallinarum* at 4.0 mg. %.

Antibacterial activity of the diphenylmethanes was markedly reduced in the presence of protein. Bactericidal end points against *Staphylococcus aureus* were abolished for all but one of the compounds (No. 27) at the maximum concentration employed (4.0 mg. %). In the case of No. 27, bactericidal activity was still demonstrated with 1% serum albumin present but the concentration required was 128 times the minimum level in plain medium (Table I).

Although the majority of compounds studied in the present series were found highly bactericidal for streptococci, none of the derivatives selected for *in vivo* testing (Nos. 6, 9, 11, 13, 16, 18, 23, 26, 33, 35, and 37) showed any marked protective action against experimental streptococcal infection in mice at the maximum doses tolerated. Number 16 administered orally at the rate of 400 mg./Kg./day proved the most effective with only 20% of mice surviving. In comparison, sulfadiazine produced 100% survival at one-half the dose employed for the diphenylmethane.

DISCUSSION

The diphenylmethanes represent an interesting group of compounds from both a chemical and biological standpoint. Aside from possessing, in general, high antibacterial properties, certain of these derivatives have been shown to exert strong teniacidal action (14), while estrogenic activity has been reported for others (15, 16).

Antifungal activity of the diphenylmethanes will be the subject of a future report. Possible use of some of the compounds as preservatives is under investigation.

SUMMARY

1. A series of 38 derivatives of diphenylmethane have been examined for antibacterial activity against a variety of organisms.

2. Activity *in vitro* appeared more or less specific for Gram-positive bacteria, 23 derivatives being bactericidal for each of the species tested in concentrations ranging from 0.004 to 4.0 mg. %, while only seven compounds were active against the respective Gram-negative organisms at levels of 0.5 to 4.0 mg. %.

3. Compounds possessing a high order of activity were markedly inhibited in the presence of protein.

4. Eleven of the derivatives were tested *in vivo* and found to be relatively ineffective against streptococcal infection in mice.

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Studies on the Absorption of Choline Chloride*,†

By CARL C. RIEDESEL‡ and H. M. HINES

Two possibilities exist for the metabolism of choline. The present study was undertaken to clarify the manner in which orally ingested choline disappears from the intestine of the rat. It was found that choline disappears from the intestine of the rat at a very constant rate, in a manner suggesting enzymatic absorption. Incubation of choline with intestinal homogenate for the same length of time as required for 39 per cent absorption (two hours) did not result in any decrease of choline concentration. When the intestinal bacteria were suppressed by means of antibiotics in the diet, choline disappeared at the same rate as in the non-treated control rats. It is concluded that choline is absorbed unchanged from the small intestine, and that intestinal bacteria play only a minor role, if any, in its disappearance.

An aspect of the problem of the metabolism of choline concerns its mode of absorption and excretion. De la Huerga and Popper (1, 2) conclude that most of the ingested choline in man is converted to trimethylamine (TMA) or its oxide (TMAO), through the action of bacteria in the small intestine and that these products are then absorbed. Wünsche (3) reports the bacterial decomposition of choline by intestinal bacteria of rats. Norris and Benoit (4) report a similar finding and note that there is a greater excretion of TMAO when choline is given orally to rats than when it is injected intraperitoneally. They explain this phenomenon on the basis of the parenterally introduced choline having had no contact with intestinal flora except for quantities which might conceivably have been excreted into the gut. On the other hand, Toda (5) and Artom and Browder (6) show that liver homogenate and liver slices are able to convert choline to TMA. Thus, while there is ample evidence indicating that choline can be converted to TMA by intestinal bacteria, it is also possible for the liver to accomplish the same task.

The present study was undertaken in an attempt to clarify the manner in which orally ingested choline disappears from the intestine of rats. It was first necessary to measure the rate of disappearance of choline from the small intestine of the rat and to learn if this disappearance could be attributed to bacterial action or to direct absorption. Incubation of choline with intestinal homogenate and determination of the rate of choline disappearance from such a mixture provided one method of ascertaining the significance of the bacterial flora in its metabolism. Another method consisted of inhibiting the bacterial flora with antibiotics and noting any effect on the rate of disappearance of choline from the intestine.

METHODS AND PROCEDURES

Materials.—All chemicals used were reagent grade. The choline chloride was dried for twenty-four hours at 90°, then stored in a vacuum desiccator over anhydrous calcium chloride. All solutions of choline chloride were made on the day of use, in 0.85% solution of sodium chloride.

The rats used were male, bred from Wistar strain albinos, averaging 150 Gm. in weight. They were maintained on a diet of Purina Laboratory Chow and water, supplied *ad libitum*. Twenty-four hours before any absorption study they were put in wire-bottom cages to prevent access to feces. Water was supplied during this time, but food withheld.

Operative.—For a typical absorption study, the rats were anesthetized with ether. Following a midline abdominal incision, a ligature was put around the small intestine at the site of the ileocecal valve and one just below the entrance of the bile duct. In later experiments, the ligature at the level of the bile duct was discontinued after it had been observed that ligation of the intestine at this position had no noticeable effect on the quantities of choline disappearing from the intestine. Of interest in respect to this latter observation is the report by Rohse and Hines (7) that there is no increase in free choline in the bile of rats following its oral administration.

With the ligature in place at the ileocecal valve, a standard dose of choline chloride was injected into the intestine. For all absorption studies, a 0.1% choline chloride solution was used, the dosage being 200 mg./Kg. of body weight. Injection was made with a 1-ml. tuberculin type syringe and a 25 gauge needle, the injection site being approximately one inch below the entrance of the bile duct. Rate of injection was sixty seconds for the entire dose. Immediately following the choline injection, the abdomen was closed in two layers, and the animals put in a warm cage, protected from drafts to recover from the anesthetic. They were kept in this cage with access to water but not to food. At the end of the respective absorption period, they were quickly killed with ether. The entire small intestine was rapidly removed, and the contents washed into an Erlenmeyer flask by repeated flushing with normal saline at 40°. The total washings were limited to 30 ml.

* Received January 23, 1953, from the Department of Pharmacology, Idaho State College, Pocatello, Idaho.

† The investigations were conducted in the Department of Physiology, State University of Iowa, Iowa City, by Carl C. Riedesel, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The investigations were supported in part by a grant in aid from Flint, Eaton and Company, Decatur, Illinois.

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Analytical.—Proteins were precipitated from solution with a slight modification of the Somogyi method (8). The hydrogen ion concentration of the solution was adjusted to *p*H 10 by addition of a 15% solution of NaOH using Hydrion paper as indicator. Bandelin and Tuschhoff (9) show that such a high *p*H prevents precipitation of contaminants such as the B vitamins and trimethylamine. The solution was filtered by suction, through a double thickness of No. 42 Whatman paper. The precipitate was washed repeatedly with small portions of warm distilled water, the final volume amounting to approximately 150 ml. The choline was precipitated with ammonium reineckate and estimated gravimetrically according to the method described by Pankratz and Bandelin (10).

Separation of Choline from TMA.—Using controlled samples, it was found possible to separate choline from TMA quantitatively by manipulation of the hydrogen-ion concentration of the solvent. Choline is precipitated from aqueous solution by an excess of ammonium reineckate at *p*H 10, while TMA remains in solution. After the choline reineckate is filtered off, adjustment of the hydrogen ion concentration to *p*H 7 will cause immediate precipitation of the TMA reineckate. The TMA can then be gravimetrically estimated.

Incubation of Choline with Gut Homogenate.—Rats which had fasted for twenty-four hours were killed by decapitation and the entire length of the small intestine removed. The excised ileum of each was finely divided in a Waring Blendor and buffered Ringer's solution was used as the dispersion medium. To each separate homogenized ileum was added the same quantity of choline chloride in solution as would have been administered to a live rat for a typical absorption study. The mixture was incubated at 38° for two hours. At the end of this time the proteins were precipitated and gravimetric determinations of choline were made as described under analytical procedure.

Inhibition of Bacterial Flora with Antibiotics.—A diet was prepared containing 0.3% aureomycin and 1% sulfaguanidine in powdered Purina Laboratory Chow. This mixture was supplied to the animals, *ad libitum* for sixty hours. Bacteriological examination of the feces showed complete suppression of the *Coli* organisms but not the *Pseudomonas* organisms. Twenty-four hours before the absorption study, all food was removed from the cage. The absorption study was conducted in the manner described under operative and analytical procedure.

RESULTS

Hourly Absorption Rate.—The hourly absorption rate for the standard solution of choline chloride was determined for periods of one, two, three and four hours. Table I summarizes the data obtained. Results are expressed in mg. of choline chloride absorbed per 100 Gm. body weight.

Incubation of Choline Chloride with Gut Homogenate.—Following the two-hour incubation period, an average of 97.1% of the added choline was recovered. No TMA could be demonstrated. The 2.9% of choline not accounted for may be attributed to experimental error since never was there TMA in sufficient quantity to be estimated gravimetrically. If the amounts of choline chloride which disappeared from the intact intestine in two hours did so because of bacterial action, then incubation of the homogenized intestine with an equivalent amount of choline for the same length of time should show a corresponding rate of disappearance of choline, and a corresponding appearance of TMA. The results are summarized in Table II.

TABLE I.—AMOUNTS OF CHOLINE CHLORIDE ABSORBED PER 100 GM. OF BODY WEIGHT IN THE RAT, DURING ABSORPTION PERIODS OF ONE, TWO, THREE AND FOUR HOURS

Period	Number of Animals	Milligrams Choline Chloride Absorbed per Hour	Percentage Absorption	Total Mg. Absorbed per 100 Gm. Body Weight[a]
1st hour	10	5.61	28.24	5.61
S.D.				1.06
2nd hour	15	3.7	38.98	7.41
S.D.				1.36
3rd hour	13	4.11	63.14	12.34
S.D.				2.79
4th hour	10	4.28	82.74	17.11
S.D.				1.82

[a] In each case the differences between the mean values of total mg. absorbed are statistically significant ($p = < 0.01$).

TABLE II.—EXPERIMENT ON THE RECOVERY OF CHOLINE CHLORIDE ADDED TO INTESTINAL HOMOGENATE AND INCUBATED AT THIRTY-EIGHT DEGREES CENTIGRADE FOR TWO HOURS

Animal Weight	Milligrams Choline Chloride Added	Milligrams Choline Chloride Recovered[a]	Percentage Recovery	Milligrams Trimethylamine Recovered
160	32	30	93.75	00
220	44	43	97.7	00
180	36	36	100.00	00
200	40	40	100.00	00
190	40	38	95.00	00
122	25	24	96.00	00

[a] Average per cent recovery of choline chloride: 97.1.

Intestinal Absorption of Choline Following Inhibition of Intestinal Flora.—During the two-hour absorption period, there was an average disappearance of 41% of the injected choline. The results are summarized in Table III.

TABLE III.—COMPARISON OF THE RATE OF INTESTINAL ABSORPTION OF CHOLINE CHLORIDE BETWEEN NONTREATED CONTROL RATS AND RATS ON A DIET CONTAINING AUREOMYCIN AND SULFAGUANIDINE

Group	Number of Animals	Mean Absorption per 100 Grams in Two Hours[a]
Controls	15	7.4
S.D.		1.36
Treated	11	8.21
S.D.		2.54

[a] The difference between the two mean absorption rates is statistically significant ($p = < 0.01$).

DISCUSSION

The fact that choline disappears from the small intestine of the normal rat at a nearly constant rate of approximately 4 mg./100 Gm. of body weight suggests a process of enzymatic absorption. This is not in agreement with those (1, 2) who believe that bacterial conversion is the prime factor in the disappearance of choline from the small intestine. If bacterial conversion were the prime factor involved,

then it should have been possible to show a corresponding rate of disappearance of choline in the incubation studies. However, incubation of choline with finely divided intestine did not bring about any loss of choline other than that which could be attributed to experimental error. Neither was there any measurable quantity of TMA formed during the two-hour incubation period. It is difficult to understand why the same bacteria which *in vivo* could cause a 39% conversion of choline to TMA should not be able to bring about a similar conversion *in vitro* during the same length of time. Furthermore, the administration of aureomycin-sulfaguanidine which was shown to depress the intestinal bacteria did not lower the rate of choline disappearance as it should have done were the bacteria the major factor involved.

There is no evidence to show that the presence of bacteria in the intestine inhibits absorption, or that absorption rates would be increased in the absence of bacteria. One may conclude, therefore, that the absorption of choline would be the same in the presence or absence of intestinal bacteria. Since it was shown that there is a constant rate of disappearance of choline and that this was not inhibited by the antibiotic given, one must also conclude that bacteria play little or no part in the disappearance of choline from the small intestine.

SUMMARY

When choline chloride was administered to rats, in doses of 200 mg./Kg. of body weight, the absorption rate from the small intestine was found to be approximately 4 mg./100 Gm. body weight per hour.

Incubation of choline with intestinal homogenate, for two hours, did not result in any appreciable decrease in choline concentration. When the intestinal bacteria were suppressed, *in vivo*, with a mixture of aureomycin-sulfaguanidine in the diet, choline disappeared from the small intestine at the same rate as in the controls.

It is concluded that choline is absorbed unchanged from the small intestine of the rat, and that the intestinal bacteria play only a minor role, if any, in its disappearance.

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Factors Affecting the Microbiological Assay of Pyridoxine in Multivitamin Products*

By A. F. NOVAK, A. R. GAMA, J. A. LIUZZO, and E. B. RUBLOFF

Effects of certain vitamins on the microbiological assay of pyridoxine when employing Difco Bacto Pyridoxine Assay Medium and a culture of *Neurospora sitophila* A. T. C. C. 9276 were studied. Potential stimulatory properties of thiamine, folic acid, biotin, *para*-aminobenzoic acid, niacin, riboflavin, vitamin B_{12}, inositol, and phytological concentrate were investigated. Except for thiamine no additional growth was noted from these crystalline vitamins. Exceptional stimulatory effects were found in the phytological concentrate. Necessity for thiamine inclusion in the medium when used for the assay of crystalline multivitamin products has been proved. This method cannot be employed for accurate pyridoxine assays when certain phytological concentrates are present, because evidence obtained indicates another factor (s), found in natural products, accentuates the growth of this organism in Difco Pyridoxine Assay Medium. The nature of this substance is now being investigated.

MOST PHARMACEUTICAL laboratories engaged in vitamin analyses prefer to employ commercial media for microbiological assays, because these products are highly standardized and the results obtained can be reasonably duplicated. Such duplications have been difficult when assaying complex vitamin preparations for pyridoxine since the presence of some substances interferes with the accuracy of the method. These circumstances have initiated numerous research projects for investigating the nutritional requirements of microorganisms employed in these procedures.

Harris (1) found that all available pyridoxine-less mutants of *Neurospora sitophila* grow better at limiting pyridoxine concentrations if thiamine

* Received February 27, 1953, from the Research Laboratories, Nutrilite Products, Inc., Buena Park, Calif.

is added, and later (2) recommended the addition of thiamine to bioassay culture media. Consequently, this modification should be required for the microbiological assay of vitamin products containing both substances, and especially when conditions exist which prevent total separation or inactivation of the interfering thiamine. Such conditions are present in vitamin products containing phytological supplements where the removal of thiamine without the concurrent destruction of pyridoxine is difficult.

This investigation was designed to study the effect of thiamine inclusion when employing Difco Bacto Pyridoxine Assay Medium for the microbiological assay of pyridoxine in multiple vitamin preparations.

Preliminary results indicated that another factor (*s*) also affected the pyridoxine determination in this medium, since the addition of thiamine alone failed to yield a growth response equivalent to that obtained upon the introduction of small quantities of a multivitamin preparation containing both crystalline vitamins and a phytological concentrate. Therefore, the possible stimulatory action of other vitamins was also investigated.

EXPERIMENTAL

Difco Bacto Pyridoxine Assay Medium (3) which is patterned after that of Stokes, Larsen, Woodward, and Foster (4) and revised by Barton-Wright (5) was used for the pyridoxine determinations. Experimental modifications of this medium and method are presented.

Weighed samples were acid hydrolyzed at *p*H 6 by boiling for two minutes, and aliquots were added to assay flasks containing the basal medium. These flasks were plugged, sterilized by autoclaving for 15 minutes at 121°, cooled, and inoculated. Sterilization was accomplished by the same method when thiamine was added to the acidic basal medium, because it was found that no significant amount of this vitamin was destroyed under these conditions. The test organism *Neurospora sitophila* A. T. C. C. 9276, which is sensitive to the three analogs of vitamin B_6, was cultured according to standard procedure. Prior to inoculation, 1 loop of spores was transferred to 50 ml. of sterile physiological saline solution. Flasks containing the test and control solutions were inoculated by introducing 1 drop of this suspension to each flask. Inoculated flasks were incubated for five days at 30°. Mycelia were steamed at 100° for five minutes in a closed water bath, removed from the flasks and rolled into pellets. The pellets were then dried at 40° for one hour, and assay results determined gravimetrically from weights of the mycelia in each flask.

To study the effect of added thiamine, two different standards were required: one containing pyridoxine only, the other containing both pyridoxine and thiamine. Typical results are presented in Table I.

Two distinct types of vitamin preparations were tested: one contained both crystalline vitamins and a phytological concentrate; the other was composed solely of crystalline vitamins. The phytological concentrate in the complex product was prepared from alfalfa, parsley, and watercress.

Pyridoxine analysis of the phytological supplemented sample gave such excessively high assay results that an investigation of the effects of this concentrate was required. One gram of the concentrate was diluted to 50 ml., and 1-ml. aliquots were added to each flask as shown in Table I.

The crystalline multivitamin sample containing 4 μg. B_6/ml. was diluted 25 times and 1-, 2-, and 3-ml. aliquots were tested. Results are noted in Table II

TABLE II.—PYRIDOXINE ANALYSIS OF CRYSTALLINE MULTIVITAMIN PREPARATION

Ml. of test sample (diluted 25 times)	1	2	3
Conc. of B_1 in μg.	15	15	15
Average mycelial wt. in mg./flask	16	23.7	26.6
Value of mycelial wt. expressed in μg. of B_6/ml.	0.156	0.160	0.157

Samples were assayed on the thiamine supplemented medium because the presence of this vitamin in the samples was responsible for higher values when an unsupplemented medium was employed in preliminary determinations. Thiamine was added at a level of 15 μg./5 ml. of assay medium, prior investigations showing this to be the maximum required.

Evidently thiamine exhibits a stimulatory effect upon the assay organism which becomes significant if not included in the assay medium when analyzing multivitamin samples of which thiamine is a constituent.

Plotted on a graph, values from Table I yield curves *A*, *B*, and *C*, respectively, in Fig. 1.

TABLE I.—GROWTH OF *Neurospora sitophila* AS AFFECTED BY SUPPLEMENTATION OF DIFCO BACTO PYRIDOXINE ASSAY MEDIUM

Supplement	Conc. of B_6 in Micrograms: 0.1	0.2	0.4	0.6	0.8	1.6
	Mycelial Wt. in Milligrams					
(A) None	2.5	5.4	8.8	16.0	23.5	27.5
(B) Thiamine 15 μg.	11	19.2	25.1	28.2	30.1	33.4
(C) Thiamine 15 μg. + Phyto. Conc. 20 mg.	28.5	40.6	42.0	43.9	43.5	43.3

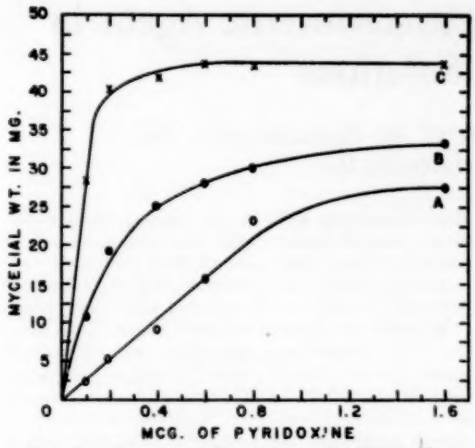

Fig. 1.—Growth of *Neurospora sitophila* in Difco Bacto Pyridoxine Assay Medium. (*A*) Thiamine free. (*B*) Thiamine supplemented. (*C*) Thiamine supplemented plus phytological concentrate.

Increased mycelial weight, obtained at low pyridoxine concentrations when the organism was grown in the medium containing both thiamine and a phytological concentrate (Fig. 1, curve *C*), was caused by the presence of some pyridoxine in the sample. However, the excessive response at higher pyridoxine levels cannot be accounted for by the pyridoxine or thiamine content of the concentrate. The maximum mycelial weight that could be caused by these vitamins is shown in curve *B*. Curve *A* illustrates the maximum response to pyridoxine in the absence of thiamine.

Analysis of the crystalline multivitamin preparation gave the typical values seen in Table II.

The average value 0.158 multiplied by the dilution factor 25 equals 3.95 μg. pyridoxine/ml. of test solution. This result represents 98% recovery. Interpolation of mycelial weights of other samples on thiamine free standard curve (*A*) produced results that ranged from 100 to 200% over the known concentrations. Evaluation of these same weights on thiamine supplemented standard curve (*B*) yielded 95–105% recovery.

Initial analyses conducted on the multivitamin preparation containing the phytological concentrate prepared from alfalfa, parsley, and watercress yielded results of such high proportions that the degree of stimulation could not be attributed to the pyridoxine and thiamine alone. Consequently investigations were extended to determine the potential stimulatory properties of *para*-aminobenzoic acid, folic acid, biotin, niacin, riboflavin, vitamin B_{12}, and inositol, which are common constituents of vitamin products.

The studies were carried out by individual addition of the above-named factors to separate flasks containing pyridoxine and thiamine. Mycelial weights in these experiments showed negligible variance from the series containing only pyridoxine and thiamine.

The increased stimulation resulting upon the addition of the preparation containing the phytological concentrate cannot be attributed to the aforementioned vitamins. Therefore, further investigation is warranted.

DISCUSSION

Neurospora sitophila grows better in Difco Bacto Pyridoxine Assay Medium at limiting pyridoxine concentration if thiamine is added. These results prove the need for supplementing this assay medium with thiamine in order to obtain accurate analyses of crystalline multivitamin preparations of which thiamine is a component. The medium must be supplemented with optimal thiamine so that it, too, does not become limiting to mycelial growth. Satisfactory results were obtained with 15 μg. of thiamine/5 ml. of assay medium. Higher levels failed to yield greater growth; amounts as high as 50 μg./5 ml. not producing any significant increase. No experiments were performed to verify the mechanism of the thiamine and pyridoxine interaction recently suggested by Harris (2).

This method was unsuccessful when employed for the analysis of pyridoxine in a multivitamin preparation containing a phytological concentrate. Considerably higher values were obtained that could not be accounted for by the addition of thiamine, folic acid, biotin, B_{12}, inositol, niacin, *para*-aminobenzoic acid, and riboflavin. Evidence suggests another factor found in natural products is necessary for the maximum growth of this organism in Difco Pyridoxine Assay Medium. This substance prevents accurate pyridoxine analyses in complex vitamin preparations containing phytological extracts.

CONCLUSIONS

1. Difco Bacto Pyridoxine Assay Medium must be supplemented with thiamine to obtain accurate pyridoxine values when analyzing crystalline multivitamin preparations containing thiamine.

2. This method cannot be employed for accurate pyridoxine assays when certain phytological concentrates are present because they contain another factor necessary for maximum growth of the organism.

3. This factor (*s*) is distinct from thiamine, folic acid, biotin, *para*-aminobenzoic acid, vitamin B_{12}, riboflavin, niacin, and inositol. The nature of this substance is now being investigated.

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Phenylethyl Alcohol as a Bacteriostatic Agent in Ophthalmic Solutions*

By JOHN H. BREWER,† SAMUEL W. GOLDSTEIN,‡ and C. BAXTER McLAUGHLIN†

NO SUBSTANCE for the preservation of ophthalmic solutions has yet been found which will answer most of the requirements for a satisfactory bacteriostatic agent. Recent difficulties with ophthalmic solutions, such as pyocyaneus-type infections, have drawn our attention to this problem. In view of the work of Lilley and Brewer (1952) (1), who demonstrated the ability of phenylethyl alcohol (PHE) to inhibit the growth of a number of Gram-negative organisms in bacteriological culture media, we investigated the use of PHE in some ophthalmic solutions.

EXPERIMENTAL

To determine the behavior of PHE in the presence of different types of ophthalmic medicinal agents and other compounds, solutions were prepared by addition of the weighed chemical or chemicals to 50 ml. of a 0.5% aqueous solution of PHE (*p*H 6.05) in a 2-fluidounce Duraglas prescription bottle. The solutions prepared and their concentrations are given in Table I. Control solutions were prepared with distilled water

After standing at room temperature (22–25°) for at least thirty days, the only solutions that showed evidence of chemical change were mercuric chloride, mercuric succinimide, and silver nitrate. It is noteworthy that the solutions of these chemicals do not require the addition of a preservative agent. They are among those that were added to the series to broaden the scope of the tests for compatibility. The only change in the control solutions occurred in the dextrose solution in which a mold grew.

After standing in an incubator at 38° for fourteen days, the only additional change was the deposition of crystals in the test solution containing phenobarbital sodium. The control solution of phenobarbital sodium in water showed a similar change.

Comparison of the results of this series of tests with results reported for other preservative agents such as phenol, chlorobutanol, benzalkonium chloride, thimerosal, methyl and propyl parabens (2), indicate that PHE in 0.5% concentration is relatively free from the problems of chemical incompatibilities with ophthalmic medicinal agents.

Since ophthalmic solutions may become contaminated with *Pseudomonas aeruginosa* (pyocyaneus), an organism which causes very serious eye infections, a representative number of eye solutions containing PHE were formulated and purposely contaminated with this organism. After periods of seven and fourteen days, attempts were made to recover the contaminating organism and determine whether or not growth was taking place.

Method.—A 24-hour infusion broth (BBL) culture of *Pseudomonas aeruginosa* was diluted in water and 1 ml. of a 1:1000 dilution was inoculated into 50 ml. of the ophthalmic solutions. At the same time dilutions were made to determine as closely as possible the number of organisms inoculated by plating into infusion agar (BBL). The solutions were then allowed to stand at room temperature until tested. Bacterial counts revealed that the inoculated ophthalmic solutions contained 36,000 organisms per ml. Our evaluations are based on whether or not subsequent samples showed these solutions to contain more or less numbers of living bacteria.

After seven days a sample of each ophthalmic solution was diluted and plated in infusion agar. Obviously, any number of organisms greater than 36,000 per ml. shows that *Pseudomonas aeruginosa* can multiply in the ophthalmic solution. After fourteen days the same test was performed. The ophthalmic solutions tested and the results are given in Table I.

DISCUSSION

Table I shows quite dramatically the population reduction in the ophthalmic solutions of *Pseudomonas aeruginosa* by phenylethyl alcohol. Distilled water without PHE contained on inoculation approximately 36,000 organisms per ml.; after seven days the count had risen to 200,000—an increase of approximately 500%.

Of 43 solutions containing PHE only two showed a very minimum of growth in seven days and were sterile at fourteen days. Twenty-six solutions without PHE were found to contain viable *Pseudomonas aeruginosa* and of these twenty-six, twenty-four contained a greater number of organisms than had been inoculated. This would seem to demonstrate conclusively that many ophthalmic solutions will support the growth of the pathogenic *Pseudomonas aeruginosa* and that a preservative such as phenylethyl alcohol is advisable.

Although a number of other solutions not containing PHE failed to support the growth of *Pseudomonas aeruginosa*, a preservative would still be needed because other contaminants may produce a medium which can support the growth of a pathogenic species.

The procedure of inoculating the solutions with so many organisms is of course quite extreme, since it is improbable that a solution would naturally become contaminated with such number of bacteria.

* Received June 29, 1953, from the Biological Research Laboratory, Hynson, Wescott & Dunning, Inc., Baltimore 1, Maryland.

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TABLE I.—THE EFFECT OF PHENYLETHYL ALCOHOL ON OPHTHALMIC SOLUTIONS CONTAMINATED WITH PSEUDOMONAS AERUGINOSA

	Conc., Per Cent	Control		PHE	
		7 Days	14 Days	7 Days	14 Days
Antipyrine	1.0	>300,000	960,000	–	–
Atropine Sulfate	1.0	>300,000	3,440,000	–	–
Benzyl Alcohol	1.0	–	–	–	–
Boric Acid	4.0	–	–	–	–
Butacaine Sulfate	1.0	3,400	10,100	–	–
Caffeine	1.0	>300,000	440,000	–	–
Chlorobutanol	0.5	–	–	–	–
Dextrose	5.6	>300,000	2,880,000	–	–
Emetine Hydrochloride	1.0	>300,000	>3,000,000	–	–
Ephedrine Hydrochloride	3.0	–	–	–	–
Ephedrine Sulfate	3.0	–	–	–	–
Fluorescein Sodium	2.0	–	–	–	–
Formin	1.0	109,000	430,000	–	–
Homoatropine Hydrobromide	2.0	>300,000	3,320,000	–	–
Hyoscine Hydrobromide	0.5	>300,000	830,000	–	–
D-Lactose	1.0	>300,000	800,000	–	–
Mercuric Chloride	1.0	–	–	–	–
Mercuric Succinimide	1.0	–	–	–	–
Metycaine Hydrochloride	2.0	>300,000	2,480,000	–	–
Nupercaine Hydrochloride	1.0	–	–	–	–
Phenacaine Hydrochloride	1.0	46,000	> 30,000	–	–
Phenobarbital Sodium	1.0	>300,000	1,350,000	–	–
Physostigmine Salicylate (with Sodium Bisulfite 1.0)	0.5	–	–	–	–
Pilocarpine Hydrochloride	1.0	>300,000	1,000,000	–	–
Potassium Iodide	1.4	34,000	> 500,000	–	–
Procaine Hydrochloride	1.0	124,000	330,000	–	–
Quinine Urea Hydrochloride (in Ringer's Solution No. 1)	1.0	< 1,000	–	1,500	–
Silver Nitrate	1.0	–	–	–	–
Silver Protein, Mild	10.0	–	–	–	–
Sodium Benzoate	1.0	–	–	–	–
Sodium Bicarbonate	1.0	>300,000	530,000	–	–
Sodium Biphosphate	1.0	120,000	150,000	–	–
Sodium Borate	1.0	–	–	–	–
Sodium Chloride	0.9	>300,000	3,000,000	–	–
Sodium Citrate	1.0	>300,000	1,920,000	–	–
Sodium Nitrate	1.5	–	–	–	–
Sodium Phosphate	1.0	>300,000	>3,000,000	–	–
Sodium Sulfadiazine	5.0	–	–	–	–
Sodium Sulfathiazole	5.0	–	–	–	–
Sodium Sulfite	1.0	–	–	–	–
Sodium Thiosulfate	1.0	>300,000	400,000	900	–
Sucrose	1.0	70,000	45,000	–	–
Tetracaine Hydrochloride	1.0	–	–	–	–
Urea	1.0	>300,000	41,000	–	–
Zinc Sulfate	1.0	–	–	–	–
Zinc Sulfate and Boric Acid	1.3 2.0	–	–	–	–
Zinc Sulfate, Boric Acid, and Sodium Chloride	0.144 2.16 0.216	–	–	–	–
Distilled Water		200,000	190,000	–	–

The PHE solutions were sterility-tested in fluid thioglycollate media after fourteen days and no growth appeared in any of these solutions.

It can be concluded, therefore, that the presence of 0.5% phenylethyl alcohol in ophthalmic solutions will prevent the growth of *Pseudomonas aeruginosa*.

Since PHE has a slight anesthetizing action and is compatible with most of the ophthalmic solutions which would require preservatives, a clinical study is under way by a group of ophthalmologists who will report on their evaluation of this material.

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Note

A Note on the Purification of Dioxane for Use in Preparing Nonaqueous Titrants*

By CATHERINE N. SIDERI† and ARTHUR OSOL

DIOXANE is often employed as the solvent medium for preparing volumetric solutions of perchloric acid for use in the titration of basic substances in nonaqueous media. It has the advantage, over glacial acetic acid similarly used, of providing a sharper potential break in potentiometric titrations involving perchloric acid and thus of affording a more sensitive means of determining the end point of a titration, as has been conclusively demonstrated by Pifer and Wollish (1).

A disadvantage of dioxane is its relatively large coefficient of expansion, which significantly influences the normality of the perchloric acid solution with changes of temperature that would have no appreciable effect on the normality of aqueous solutions. This temperature effect may be compensated for readily either by standardizing the solution at the temperature at which it is employed or by applying a correction based on the coefficient of expansion of the solution. Another, and more serious, disadvantage of dioxane is that it generally contains impurities which cause more or less coloration of the solution a short time after perchloric acid is added; sometimes the solution is a very dark brown.

As pointed out in the review article by Pifer, Wollish, and Schmall (2), dioxane may be purified by several methods, as by distillation over sodium, or by mixing it with Amberlite IRA-400 and filtering. The former method is relatively bothersome, and the latter may result in some minute particles of the basic resin passing into the filtrate and subsequently reacting with the perchloric acid.

We have observed that a very simple procedure served to purify all samples of dioxane recently employed, to a degree that no coloration whatsoever occurred in the solutions of perchloric acid we prepared. The purification procedure consists in adding asbestos, of the type employed in preparing filtration mats (we used the "Powminco" brand), in the proportion of approximately 20 Gm. per liter of dioxane, shaking the mixture occasionally during an hour or so, then filtering it through a plug or pad of asbestos. Even with those lots of untreated dioxane which produced a deep brown coloration when perchloric acid was added, colorless solutions were obtained when the dioxane was treated as described. Our experience has been confirmed by Pifer, Wollish, and Schmall (2).

Since some lots of dioxane cause less coloration with perchloric acid than do others, the amount of asbestos employed may in such cases be reduced, and the treatment simplified to that of passing the dioxane through a layer of asbestos; also, the spent asbestos may be prepared for re-use by washing with water, drying, and igniting.

* Received June 23, 1953, from the Chemistry Department of the Philadelphia College of Pharmacy and Science, Philadelphia.

This paper is the report of an observation made in the course of research on nonaqueous titration methods performed by Catherine N. Sideri in partial fulfillment of requirements for the degree of Doctor of Science, at the Philadelphia College of Pharmacy and Science.

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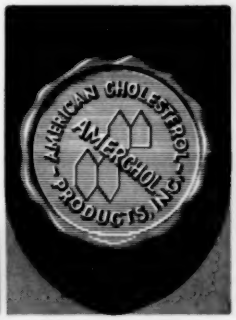

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